THE EFFECT OF SEQUENTIAL EXPOSURE TO ANTIGENICALLY DRIFTED STRAINS OF INFLUENZA ON ANTIBODY SPECIFICITY AND ANTIBODY MEDIATED PROTECTION

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ABSTRACT

THE EFFECT OF SEQUENTIAL EXPOSURE TO ANTIGENICALLY DRIFTED STRAINS OF INFLUENZA ON ANTIBODY SPECIFICITY AND ANTIBODY MEDIATED PROTECTION

Susanne L Linderman

Scott E Hensley

Antibodies (Abs) elicited by influenza viruses often bind with higher affinities to past influenza strains than to the current strain. This has been associated with the hypothesis of "original antigenic sin" which postulates that B cell clones elicited by prior exposure are recalled at the expense of generating a strain-specific response to the current strain. It is clear that prior exposures to influenza viruses focus the response on conserved epitopes of influenza's hemagglutinin (HA) glycoprotein. Here, pre-exposure to influenza is shown to affect Ab fine specificity and Ab mediated protection in mice, ferrets, and humans. Sequential vaccination of mice with antigenically drifted strains of influenza virus elicited a large proportion of cross-reactive Abs, but also recalled Abs that have a higher affinity for the priming strain while binding to the same region of HA with different fine specificities. These fine specificities were modified by somatic hypermutation, which affected the degree of cross-reactivity. Surprisingly, Abs that bound with relatively low affinities to an antigenically drifted strain effectively neutralized the drifted strain following passive transfer. This indicates that Abs expressed by recalled B cell clones share some level of cross-reactivity between antigenically drifted priming and recall strains. This was also observed by measuring the reactivity of sera to HA of antigenically drifted influenza strains. Similarly, ferrets and humans sequentially exposed to influenza viruses had Ab repertoires that were focused on epitopes conserved between recent strains and earlier strains that circulated during their childhood. As influenza viruses continually mutate, humans had Ab repertoires that were focused on different regions of the HA, potentially due to unique pre-exposure histories. A recently acquired HA mutation abrogated binding of Abs predominantly found in middle-aged adults that were unusually susceptible to influenza infection during the

2013-2014 influenza season. These findings highlight that while weakly cross-reactive Abs can protect against antigenically drifted strains, shifting the Ab repertoire to partially conserved sites can focus the response on regions of the virus that undergo continual antigenic drift. Recall of cross-reactive Abs can thus result in protection or susceptibility to influenza, depending upon the exposure history and Ab fine specificities.

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LIST OF ABBREVIATIONS

Abs	Antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
BCR	B cell receptor
B/Lee	B/Lee/1940
BPL	Beta-propiolactone
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CCL19	Chemokine (C-C motif) ligand 19 (ELC, CKb11, MIP-3b, SCYA19)
CCL21	Chemokine (C-C motif) ligand 21 (ECL, SLC, CKb9, TCA4, SCYA21)
CCR7	Chemokine (C-C motif) receptor 7 (CD197, EBI1)
CD154	CD40L
CD40	TNF receptor superfamily member 5
CD45R	Protein tyrosine phosphatase receptor type C (B220)
CD80	B7-1
CD86	B7-2
CDC	Centers for Disease Control and Prevention
CDR	Complementarity determining region
CHOP	Children's Hospital of Philadelphia
CTLs	Cytotoxic T lymphocytes
D	Diversity
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fc	Immunoglobulin constant region
FDC	Follicular dendritic cell
FR	Framework region
GISN	Global Influenza Surveillance Network
GISRS	Global Influenza Surveillance and Response System
HA	Hemagglutinin
HAI	Hemagglutination inhibition assay
HAU	Hemagglutination unit
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIV	Human immunodeficiency virus
IMGT	International immunogenetics information system
i.p.	Intraperitoneal
i.v.	Intravenous
J	Joining
J1	A/Puerto Rico/08/1934-J1
LCMV	Lymphocytic choriomeningitis virus
mAbs	Monoclonal antibodies
MBCs	Memory B cells
MDCK	Madin-Darby canine kidney
MEM	Minimum essential medium

MHC II	Major histocompatibility complex II
NA	Neuraminidase
NIH	National Institutes of Health
OAS	Original Antigenic Sin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
pH1N1	Pandemic H1N1 A/California/07/2009
PNPP	p-nitrophenyl phosphate
PR8	A/Puerto Rico/08/1934
RIA	Radioimmunoassay
RAG	Recombination-activating genes
RNA	Ribonucleic acid
S12a	A/Puerto Rico/08/1934-S12a
SD	Standard deviation
SEM	Standard error of the mean
sH1N1	Seasonal H1N1
TAE	Tris-acetate-EDTA
TCID50	50% Tissue culture infectious dose
TFH	T follicular helper cell
TLR	Toll-like receptor
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
V	Variable
VLRB	Variable lymphocyte receptor B
WHO	World Health Organization
WT	Wild type

CHAPTER 1: INTRODUCTION

Influenza causes an annual disease burden throughout the world, and has for centuries. Influenza-like epidemics have been recorded throughout history, though before the isolation of the causative influenza virus, these epidemics were often considered to be new diseases, particularly since they frequently overcame previously acquired immunity (Francis, 1953; Smith et al., 1933). In 1562, Sir Thomas Randolph, the Ambassador of Queen Elizabeth I to the Court of Mary, Queen of Scots, recorded in a letter, "May it please your Honour immediately upon the Queen's arrival here, she fell acquainted with a new disease that is common in this town, called here the New Acquaintance, which passed also through her whole Court, neither sparing lord, lady, nor demoiselle, not so much as either French or English" (Strickland, 1903). Not all pandemics were equal in their epidemiological distribution though. For instance, English reports of the pandemic of 1782 describe that children and the elderly were less affected than middle-aged adults (Francis, 1953).

We now know that influenza viruses frequently cause epidemics because they can rapidly change, either by acquiring mutations or by gene recombination. In addition, we know that serum components known as antibodies (Abs) can protect against infection, and that the Ab repertoire is diverse and can continue to evolve over time (Briney and Crowe, 2013; Von Stabsarzt and Kutasati, 1890). In chapter two, we will describe how sequential exposure to variant influenza strains affects the fine specificity of influenza-specific Abs and influences protection against infection in mice. In chapter three we will address how pre-exposure history in humans affects the Ab repertoire and may influence susceptibility to disease. But first in chapter one, we will review

immunological and virological studies that shape our current knowledge of the B cell response to influenza and outline the larger immunologic context of the ensuing experimental questions.

A diverse repertoire of B cells express antibodies

B cells are lymphocytes that have successfully rearranged their immunoglobulin locus to express functional Abs. Functional Abs consist of four chains: two heavy chains and two light chains (or up to 6 multiples of that) (Cattaneo and Neuberger, 1987; Edelman, 1973). Each heavy and light chain of an antibody molecule has a variable region and a constant region. The variable regions are formed by gene recombination. In the heavy chain locus, the heavy chain variable region is created by sequential rearrangements involving one V (variable), one D (diversity), and one J (joining) gene segment. The light chain variable regions consist of one V gene rearranged to one J gene (Early et al., 1980; Hozumi and Tonegawa, 1976). In addition to the combinatorial diversity that is generated by the random rearrangement of these gene segments and by the independent pairing of the heavy and light chains, additional variability is introduced at the junctional sequences between the different gene regions as nucleotides can be added or lost during RAG (recombination-activating gene) mediated recombination (Alt and Baltimore, 1982). This junctional diversification can include the addition of palindromic sequences by RAG (P-addition), the addition of random nucleotides by the enzyme terminal deoxynucleotidyl transferase (N-addition), and the exonucleolytic nibbling by non-homologous end joining machinery (deletions). Due to the high degree of junctional diversity, the resulting rearrangement may result in a non-functional rearrangement (due to shifts in the reading frame or the generation of termination codons). Should recombination lead to a nonproductive rearrangement, the second

immunoglobulin allele can be rearranged. Usually only one allele is expressed (allelic exclusion), so that B cells usually express only one kind of antibody molecule (Vettermann and Schlissel, 2010). B cells harboring Abs that exhibit binding to self-antigens in the bone marrow are eliminated or disabled in a process known as negative selection (Hartley et al., 1991; Vettermann and Schlissel, 2010). In some cases, self-reactive B cells may be saved by modifying the specificity of the antibody through additional gene rearrangement, a process known as receptor editing (Luning Prak et al., 2011).

In addition to the variable region genes, each Ab heavy chain also has a constant region gene that determines the isotype (eg. IgM, IgD, IgG, IgA, or IgE). The isotype produced by a particular B cell can change over time through class switch recombination and imparts important functional properties to Ab activity (Stavnezer et al., 2008). For instance, IgA can be transported to mucosal surfaces such as the gut, and can be transferred into secretions such as saliva and milk while IgG can be transported across the placenta and provide early protection to infants (Garty et al., 1994; Palmeira et al., 2012; Van de Perre, 2003). Ab heavy chain isotypes also differ in their ability to bind to different Fc receptors which, in turn, can either enhance or suppress the immune response. This can be further modified by differential glycosylation of the Fc region, thus altering the type of receptor to which the Fc region can bind (Pincetic et al., 2014). In addition, changes in isotype have been shown to affect both avidity and affinity to antigen (Cooper et al., 1993; Tudor et al., 2012). Abs can be secreted as soluble proteins or expressed on the cell membrane as the B cell receptor (BCR) when alternatively spliced to include a transmembrane domain.

The BCR has the ability to bind to particular structural epitopes, and can transduce a signal across the cell membrane upon binding through its association with the Igα and Igβ coreceptors (Sanchez et al., 1993). This initial signal leads to significant changes in the cell that affect antigen uptake, processing, presentation on major histocompatibility complex II (MHCII), costimulatory molecule expression, and homing which make B cells effective antigen presenting cells. Naïve B cells circulate through the blood and lymph and home to secondary lymphoid organs including lymph nodes and the spleen (Butcher and Picker, 1996). Upon encountering antigen, B cells upregulate the chemokine receptor CCR7 which binds to the chemokines CCL19 and CCL21 which are expressed at the interface of the B and T cell zones in secondary lymphoid organs thus bringing the B cell in closer proximity to T cells (Reif et al., 2002). In addition, BCR binding also upregulates costimulatory molecules such as CD86 and CD80 which help the B cell stimulate CD4 helper cells (Lenschow et al., 1994). Activated B cells also upregulate CD40 which binds to CD154 on activated T cells (Lee et al., 2003). Not all B cells require T cell help. Some antigens have been shown to activate B cells in the absence of T cell help, but in conjunction with other activating signals such as Toll-like receptor (TLR) binding and extensive BCR-crosslinking of repetitive epitopes (Vos, 2000).

The germinal center response

Activated B cells can follow several different paths. They can become rapidly dividing plasmablasts that downregulate their surface immunoglobulin and produce secreted antibodies, they can become plasma cells that secrete even larger quantities of Ab, or they can become memory B cells (MBCs). Activated B cells can also enter germinal centers, where they can either die or give rise to MBCs or plasma cells.

Germinal centers are located in peripheral lymphoid organs. The established germinal center is polarized into two zones: the light zone and the dark zone (Röhlich, 1930). The dark zone is commonly associated with increased cell proliferation, somatic hypermutation, and class switch recombination resulting in a greater diversity of Ab specificities (Jacob et al., 1991b; Muramatsu et al., 2000; Victora et al., 2010). However, germinal center B cells can also divide in the light zone which is characterized by the presence of additional cell types such as follicular dendritic cells (FDC), follicular helper T cells (TFH), and macrophages (Allen et al., 2007). It is thought that high affinity Abs are preferentially selected in the light zone by competing for antigen presented on FDCs and/or competing for help from TFHs and that this results in enhanced proliferation and survival (Anderson et al., 2009; Gitlin et al., 2014). In addition to competing with other clones for antigen, individual cells may also need to compete with secreted Ab for particular epitopes, thus raising the bar for high affinity clones (Zhang et al., 2013). MBCs emerging from a germinal center response can be long-lived and quiescent until reactivated (Jacob et al., 1991a; Toyama et al., 2002).

While the mechanism for B cell fate determination is not yet clear, recent studies have demonstrated that the kinetics of MBCs and long-lived plasma cell development in the germinal center differ. Using bromodeoxyuridine (BrdU) labeling at different time points during a germinal center response, it has been determined that the majority of MBCs arise from divisions occurring during the early germinal center response, whereas most long-lived plasma cells arise from divisions occurring later on in the response (Weisel et al., 2016). In addition, destruction of germinal centers at an intermediate time point of the response by anti-CD154 Ab administration has a greater effect on the long-lived plasma cell population than on the MBC population (Weisel et al., 2016). These

findings suggest that while the germinal center selects for high affinity clones over time, it also produces a greater diversity of MBC specificities early on.

Memory B cell responses

MBCs can be long-lived and remain quiescent in the absence of antigen (Maruyama et al., 2000). However, upon antigen stimulation, MBCs can rapidly become activated and can differentiate into Ab secreting plasma cells or reenter a germinal center (Deenick et al., 2013; McHeyzer-Williams et al., 2015). MBCs can be divided into subsets by several criteria and these subsets of MBCs appear to be poised to follow different fates upon reactivation. When divided by isotype, IgM+ MBCs are more likely to reenter germinal centers whereas IgG+ MBCs are more likely to differentiate into Ab secreting plasma cells (Dogan et al., 2009). MBCs can also be divided into subsets based on their expression of CD80 and PD-L2 with double positive MBCs (CD80+, PD-L2+, predominantly but not exclusively IgG+) more likely to differentiate into Ab secreting plasma cells, and double negative MBCs (CD80-, PD-L2-, predominantly IgM+) more likely to form germinal centers (Zuccarino-Catania et al., 2014). Interestingly, the double negative MBC subset generally also has a lower degree of somatic mutation (Tomayko et al., 2010), suggesting that MBCs with less somatic mutation are more likely to enter germinal centers and may be precursors of the higher affinity double positive MBC subset. While it has been shown that the proportion of these MBC subsets is affected by precursor frequency, how the mode of exposure or the pathogen itself affects the overall makeup of the MBC compartment remains to be determined (Tomayko et al., 2010). This could have implications for the quality and kinetics of MBC responses as well as for the degree of Ab evolution over time.

MBCs have advantages over naïve B cells in their sensitivity to reactivation, proliferation, higher precursor frequency, and reduced requirement for T cell help. There are many intrinsic differences between naïve and MBCs that may contribute to a larger, more rapid Ab response during a secondary exposure (Tomayko et al., 2008). Human MBCs express higher levels of costimulatory molecules such as CD80 and CD86, which may lead to enhanced stimulation during a secondary response (Liu et al., 1995). MBCs also initiate proliferation more rapidly and more frequently upon antigenic stimulation than naïve B cells (Tangye et al., 2003). In addition the increased proportion of class-switched B cell clones in the MBC repertoire may affect the quality of the recall response; studies comparing B cell clones with identical specificities but different isotypes demonstrate that the IgG membrane tail increases Ab production and clonal expansion and reduces cell loss (Martin and Goodnow, 2002). As a primary response expands clonal populations of antigen-specific B cells, antigen-specific MBCs are also present at a higher precursor frequency than naïve B cells (Burnet, 1957; Tas et al., 2016). Finally, while T cell-dependent MBCs specific for soluble monomeric antigens appear to require T cell help for additional differentiation, virus-specific MBCs have been shown to differentiate into plasma cells in the absence of T cell help (Hebeis et al., 2004; Hilbert et al., 1989).

Influenza virus

Influenza viruses are enveloped single-stranded negative sense RNA viruses with 8 RNA segments (McGeoch et al., 1976). The segmented nature of the viral genome is important, as reassortment of RNA segments from 2 or more strains can occur (Marshall et al., 2013). Reassortment can lead to the development of a new subtype of influenza virus which, when infecting humans, can result in an antigenic shift.

The envelope surface proteins include hemagglutinin (HA), neuraminidase (NA), and M2 proteins (Lamb et al., 1985). HA is a homotrimer initially synthesized as the precursor HA0 but is further cleaved into two subunits, HA1 and HA2, which remain linked by disulfide bonds (Klenk et al., 1975; Lazarowitz and Choppin, 1975; Skehel and Waterfield, 1975). Once cleaved, HA aids in viral attachment and entry into a cell. It binds to sialic acids linked to galactose by predominantly 2-3 or 2-6 linkages. The frequency of these different types of linkages in different species is thought to contribute to infectivity of viral strains in different species (Ibricevic et al., 2006; Matrosovich et al., 2000). Upon viral uptake by endocytosis, a drop in pH in the endosome induces a structural change in the HA which allows the viral membrane to fuse with the endosomal membrane, allowing the contents of the virion to be released into the cytosol (Gray and Tamm, 1998). NA aids in viral release by cleaving sialic acid linkages thus preventing the HA of newly formed virions from remaining bound to the infected cell (Palese et al., 1974). M2 is a proton-selective ion channel that is thought to control the pH of the virion in the endosome before membrane fusion, and also in the golgi apparatus to maintain HA conformational stability (Takeda et al., 2002; Takeuchi and Lamb, 1994). It also binds to the M1 protein, affecting virion structure, and is required for influenza virus induced inflammasome activation (Ichinohe et al., 2010; McCown and Pekosz, 2006).

Most influenza virus neutralizing Abs bind the HA protein and neutralize either by blocking attachment, preventing fusion, or mediating other Ab mediated mechanisms of protection such as antibody-dependent cell-mediated cytotoxicity (ADCC) (DiLillo et al., 2014; Ekiert et al., 2009; Knossow et al., 2002). However, due to the influenza virus's error-prone polymerase, and the mutational permissiveness of HA, mutations can rapidly become fixed in the circulating viral population. This gradual accumulation of mutations

over time is known as antigenic drift. The membrane distal HA head domain (in contrast to the more membrane proximal stalk or stem domain) is particularly permissive of mutation and these mutations can significantly affect the binding of previously elicited Abs (Heaton et al., 2013). Because of this loss in Ab specificity, a previously protected individual can become susceptible to newly drifted strains of influenza virus. In addition, HA mutations not only affect the antigenic properties of the HA, but also its affinity for sialic acid (Hensley et al., 2009).

Influenza virus surveillance and vaccine strain selection

As influenza viruses are constantly acquiring mutations, new mutant strains that abrogate Ab binding in at least a subset of the human population can arise and supplant circulating strains that are more easily neutralized by preexisting immunity. To monitor these constant changes, the World Health Organization (WHO) has developed a surveillance system known as the Global Influenza Surveillance and Response System (GISRS) (formerly known as the Global Influenza Surveillance Network or GISN). In this system, thousands of viral isolates collected from influenza virus infected individuals are screened for their genetic and antigenic properties. To determine whether a virus is antigenically distinct compared to previously circulating strains, the clinical isolates are tested by hemagglutination inhibition assay (HAI). The HAI assay tests the ability of antisera to inhibit influenza virus's ability to hemagglutinate red blood cells. The antisera used in these screens are primarily collected from ferrets that were infected with the previously circulating strain, or in more limited cases antisera collected from humans are used. The WHO makes recommendations on which strains should be included in the next vaccine based on whether or not the antisera elicited by the previous year's vaccine are still able to inhibit hemagglutination mediated by the new isolate. The WHO also

considers results from microneutralization assays completed with a subset of clinical viral isolates. These assays measure neutralizing Abs against both the HA head and stalk (Network, 2011).

Original Antigenic Sin

Studies of influenza virus-specific Abs in human sera demonstrate that the Ab repertoire differs among different age groups and that the specificity of human sera is skewed toward those strains that would have circulated when an individual was a child (Davenport and Hennessy, 1956; Davenport et al., 1953; de St.Groth and Webster, 1966a; Francis, 1955). This has been recapitulated in multiple animal models (de St.Groth and Webster, 1966b; Kim et al., 2009; Virelizier et al., 1974; Webster, 1966). As structural analogues can recall previously elicited B cell clones (Deutsch et al., 1973; Eisen, 1969), it has been hypothesized that B cell clones specific for the initial strain are recalled by antigenically related strains experienced later in life (de St.Groth and Webster, 1966a; Li et al., 2013; Wrammert et al., 2011). However, Abs expressed by B cell clones recalled by a structural analogue frequently bind with a higher affinity to the priming antigen (Eisen, 1969). Though these B cell clones are often cross-reactive (de St.Groth and Webster, 1966b; Eisen, 1969; Fish et al., 1989; Webster et al., 1976), this observation was termed original antigenic sin (OAS) as the Abs expressed by the recalled clones have a higher affinity for past strains while the magnitude of strain-specific Ab titers specific for the more recent strain is frequently reduced (Francis, 1955). This reduction in strain-specific Ab titers to the more recent strain can be limited in a number of ways. For instance, while sequential infection with live virus leads to a reduction in strain-specific Ab titers that are specific exclusively for the more recent strain, sequential vaccination with inactivated virus does not result in an Ab titer

reduction (de St.Groth and Webster, 1966b; Kim et al., 2009). Whether this is due to a difference in dose, or due to a difference in the site of exposure is not yet understood. It is clear however, that the viral titers after a secondary infection are often lower in animals pre-exposed to antigenically drifted strains, suggesting that cross-reactive protection that reduces viral titers to the newer strain most likely contributes to a reduction in a novel Ab response (Kim et al., 2009). Many aspects of OAS might therefore simply result from decreased antigen loads during secondary exposures.

Evidence for OAS in other model systems

OAS has also been described in other systems using structurally related antigens. The haptens arsanilic acid and sulfanilic acid are two such structural analogues. While immunization with arsanilic acid elicits the dominant expression of Abs encoded by a particular heavy chain variable region, immunization with sulfanilic acid does not elicit the expansion of these B cell clones unless mice have been primed with arsanilic acid. Additionally, these Abs bind with a higher relative affinity to arsanilic acid than to sulfanilic acid (Fish et al., 1989). OAS has also been described after sequential exposure to serologically distinct variant bacterial strains (termed serovars). Studies describing sequential exposure to different serovars of *Chlamydia trachomatis* demonstrate a boost in Ab titers to the priming serovar, an increase in cross-reactive serum titers, and an altered Ab repertoire after heterologous exposure (Berry et al., 1999). OAS has also been demonstrated after sequential exposure to different subtypes of dengue virus and is also hypothesized to play a role in the evolution of the antibody response to HIV (Ciupe et al., 2011; Halstead et al., 1983).

An OAS-like phenomenon has also been described for cytotoxic T lymphocytes (CTLs) in response to sequential infection with variant strains of lymphocytic choriomeningitis virus (LCMV), dengue virus, and HIV (Klenerman and Zinkernagel, 1998; Mongkolsapaya et al., 2003; Ueno et al., 2007). In the cases of LCMV and dengue virus, activated CTLs are found to be directed against the priming strain rather than a new variant. In the case of HIV, the variant-specific CTL population expands but has reduced cytotoxic activity compared to CTLs specific for the priming strain.

Development of universal influenza vaccines with sequential exposure strategies.

According to the doctrine of OAS, the Ab repertoire is skewed towards clones that have been previously elicited by earlier circulating strains. This has been thought to result in both positive and negative effects. On the one hand, it can skew the Ab repertoire to more conserved sites. Should these conserved sites be broadly conserved and maintained, due to structural or functional constraints, Abs specific for these sites have the potential to be more broadly cross-reactive and may provide protection against a broader repertoire of viral strains. On the other hand, should the Ab response be shifted to epitopes that are not neutralizing or that are not broadly conserved, this could reduce protection against other strains. In addition, the reduction in Ab titers specific for newly formed epitopes can reduce protection against newly drifted strains. Shifting Ab immunodominance to highly conserved sites is the target of several experimental vaccine regimens designed to broaden and increase protection against rapidly mutating and diverse viral populations (Krammer et al., 2013; Malherbe et al., 2011; Wang et al., 2010; Wei et al., 2010). While sequential vaccination has been successfully used to increase the proportion of Abs elicited to conserved regions of the HA, this has been a

transient effect that is lost upon re-exposure to an antigenically related strain (Andrews et al., 2015; Ellebedy et al., 2014).

Antigenic properties of influenza hemagglutinin and the description of antigenic sites.

The earliest documented way of experimentally determining if two influenza virus strains were antigenically similar was to test if one strain elicited a protective response against another, either by challenging pre-infected ferrets with a second strain, or by incubating a viral strain with antisera collected from previously infected ferrets or humans and then testing its infectivity (Smith et al., 1933).

With the discovery of the hemagglutination property of influenza viruses, the HAI assay became a widespread method for testing the degree of antigenic difference (Hirst, 1941). Unfortunately, as the HAI assay only measures the ability of serum to inhibit influenza virus's ability to hemagglutinate red blood cells, it only measures the subset of the Ab repertoire that inhibits hemagglutination. For example, stalk-specific Abs as well as select Abs specific for the HA head domain fail to inhibit virus mediated hemagglutination, and are therefore not measured by the HAI assay. However, this was not apparent during early studies of the influenza antibody response, as the binding footprints of HA-specific Abs and their effect on hemagglutination as well as neutralization were unknown.

Several approaches were taken to determine the regions of the HA where antibodies bind, otherwise known as the antigenic sites of HA. The structure of the H3 HA from A/Hong Kong/1968 was solved in 1980 (Wilson et al., 1981). Using sequences of previously circulating H3N2 viruses as well as laboratory variants that had been

isolated after incubation with monoclonal antibody (mAb) or most-avid-fraction Ab, amino acids that differed between isolates were mapped to the HA structure (Wiley et al., 1981). The combination of the sequence and structural data led to the description of 4 different antigenic regions of the A/Hong Kong/1968 H3 HA (A, B, C, and D). Not all mutations were considered to be antigenically relevant with some characterized as antigenically neutral based on their location on the HA structure. A mutation may be antigenically neutral as any mutation may be positively selected for enhanced viral fitness or structural stability independent of Ab binding, or may accumulate due to the stochastic nature of viral mutation. In addition, some mutations, particularly those located at the interface of the HA subunits, may indirectly affect Ab binding by altering more distant epitopes, and may therefore not be in an antibody footprint themselves. However, this does not preclude the possibility that apparently neutral mutations may be antigenically relevant in a different context, such as in individuals with different antibody repertoires or due to the loss of a glycosylation site (Skehel et al., 1984). As this process of delineating antigenic sites depends on mapping HA mutations, it may also overlook residues in potential antibody footprints that are functionally constrained and therefore not mutated in infectious virus, or non-neutralizing Abs that do not positively select for HA mutations.

The H1 HA antigenic sites were initially mapped differently. Rather than mapping mutations directly to the HA structure and dividing these into different regions based on structural components, viral mutants isolated after incubation with neutralizing mAb were grouped based on the binding characteristics of a panel of virus-specific mAbs developed after primary or secondary exposure to A/Puerto Rico/08/1934 (PR8) in BALB/c mice (Gerhard et al., 1981). Viral mutants that abrogated binding of a particular

set of mAbs were grouped separately from viral mutants that bound to those mAbs but failed to bind to others. Four antigenic sites were initially assigned based on antibody binding rather than topology. These were known as the Sa, Sb, Ca, and Cb sites. These are not necessarily discrete sites, as some mAbs overlapped multiple antigenic sites (Gerhard et al., 1981; Lubeck and Gerhard, 1981). Once the viral mutants in each antigenic site were sequenced and mapped to the H3 HA structure, the antigenic sites were found to group together in different topographically distinct regions of the HA head group with the Sa and Sb sites located on the distal end of the head and the Ca (further subdivided into the Ca1 and Ca2 subgroups) and Cb sites located more proximally to the membrane (Caton et al., 1982).

Recent advances in sequencing technology have aided the continued description of HA antigenic sites. Antigenicity of particular residues as described by potential codon bias towards diversification, positive selection of amino acids, and entropy and likelihood ratios in large cohorts of HA sequences have correlated with the previously described antigenic sites, but new potential antigenic residues have also been identified (Bush et al., 1999; Huang et al., 2012; Plotkin and Dushoff, 2003). In addition, co-crystallizations of mAb bound to HA has elucidated new binding sites, particularly in highly conserved regions where mutations are rare or impossible due to functional constraints (Dreyfus et al., 2013). This is of particular importance to the understanding of antigenic sites as Abs that bind to highly conserved, rarely mutated sites are potentially broadly neutralizing and may protect against a wider diversity of strains despite antigenic drift. The original naming schemes of the antigenic sites described for the A/Hong Kong/1968 and PR8 HA are often appropriated for other viral strains, and new naming schemes for particular strains have been proposed (Huang et al., 2012). However, caution should be exercised

when applying these defined antigenic sites to new strains as the HA structure has evolved over time, and our understanding of HA antigenicity continues to advance.

B cell Immunodominance

While most PR8-specific mAbs can be mapped to the dominant antigenic sites previously mapped on the HA head domain, the site dominance differs between the early primary and secondary responses. For instance, approximately 50% of the early primary mAbs in BALB/c mice are specific for the Cb site and many of these mAbs share a limited set of light and heavy chain regions (Kavaler et al., 1990). However, these clones (that can be recognized by an Ab specific for a light chain-associated idiotope known as 23-1 Id) are not recalled by a secondary exposure (Kavaler et al., 1991). Why this particular set of epitopes is immunodominant in the primary response and not after a secondary response is not known. More recent studies on this particular specificity suggest that the early PR8 Cb response relies predominantly on extrafollicular responses, although low numbers of 23-1 Id+ cells with germinal center markers (CD45Rhi CD38lo CD24hi) are also evident (Rothaeusler and Baumgarth, 2010).

Why epitopes of the HA head are more immunodominant than more conserved epitopes located in the stalk region of the HA is unknown. Some have hypothesized that the subdominance of stalk epitopes may be due to steric hindrance as viral glycoproteins are clustered together tightly at the viral surface. This steric hindrance is maintained when HA is solubilized by bromelain mediated cleavage from the membrane as the HA molecules aggregate and form rosettes (Kawasaki et al., 1983). Interestingly, recent research in lampreys has shown that even the structurally unrelated variable lymphocyte receptor B (VLRB) response is targeted towards the HA head when these jawless

vertebrates are immunized with inactivated influenza virus (Altman et al., 2015). The immunodominance of the HA head region is therefore not immunoglobulin or species specific, but may rather be due to some inherent chemical or structural components. Multiple strategies to overcome subdominance have been proposed, from sequential vaccination as described above, to expanding the B cell repertoire by adding adjuvants to the immunogen (Clegg et al., 2012; Goff et al., 2013; Khurana et al., 2011a).

Experimental Questions

The theory of OAS is somewhat paradoxical to the theory of B cell clonal selection as it posits that antigenically drifted strains of influenza recall B cell clones specific for an earlier circulating strain that are not well adapted for the present strain. While this has been documented in both humans and various animal models, the fine specificity of OAS Abs that have a higher affinity for the priming antigen than the recall antigen, and their potential role in protection against the current strain have not been carefully described. We address this question in chapter two by sequentially immunizing mice with antigenically drifted strains of influenza. We analyze mouse sera, and generate hybridomas from sequentially immunized mice. We map the fine specificities of the mAbs derived from these hybridomas by testing their binding by ELISA to a large panel of mutant viruses to determine how pre-exposure history affects the specificity of the Ab repertoire. To determine the role of OAS Abs in protection against the contemporary influenza strain, we test the ability of mAbs to neutralize influenza virus infection *in vivo* by passive transfer experiments in mice. Our data suggest that the threshold of cross-reactivity required to recall B cell clones that are specific for previous strains is low, but that it has an effect on the fine specificity on the Abs that are recalled and that these OAS Abs contribute to protection against an antigenically drifted strain.

This is important, as it changes our understanding of OAS and how the Ab repertoire is affected by sequential exposure to influenza viruses.

In chapter three, we complete a series of experiments to determine how previous influenza exposures shape human Ab responses against new viral strains. It has been shown that individuals from different age groups can have Ab repertoires that are focused on different antigenic sites and this is hypothesized to be due to different influenza virus pre-exposure history (Davenport et al., 1953). We hypothesize that this might contribute to age group specific susceptibility to influenza strains that have acquired mutations that abrogate binding of Abs prevalent in a particular age group. In the 2013-2014 influenza season, middle-aged adults were affected by an unusually high influenza disease burden. We hypothesize that this is due to the recent acquisition of a mutation that abrogates binding to Abs prevalent in middle-aged adult sera. To test this theory, we analyze human sera from healthy human blood donors, humans vaccinated with the A/California/07/2009 strain (pH1N1), and ferrets sequentially infected with circulating H1N1 strains. These experiments not only address questions regarding current influenza surveillance methodology, but also contribute to our understanding of how influenza repertoire differences in humans affect protection to antigenically drifted circulating strains of influenza.

CHAPTER 2: 'ORIGINAL ANTIGENIC SIN' ANTIBODIES ARE A VALUABLE COMPONENT OF SECONDARY IMMUNE RESPONSES TO INFLUENZA VIRUSES

Summary

Human Abs elicited by influenza viruses often bind with a high affinity to past influenza virus strains, but paradoxically, bind poorly to the viral strain actually eliciting the response. These Abs have been referred to as 'original antigenic sin' Abs. Using a mouse model, we find that OAS and non-OAS Abs target the same general region of the influenza HA and that somatic hypermutation can modify the degree of cross-reactivity of OAS Abs. Surprisingly, although OAS Abs bound with very low affinities, some were able to effectively neutralize an antigenically drifted viral strain following passive transfer *in vivo*. Taken together, our data indicate that OAS Abs share some level of cross-reactivity between priming and recall viral strains and that these Abs can be protective when recalled into secondary immune responses.

Introduction

Influenza viruses continuously acquire mutations in antigenically important regions of the HA and NA proteins through a process termed 'antigenic drift'. Single HA mutations can dramatically reduce Ab recognition of influenza viruses (Caton et al., 1982). Although influenza infections lead to strain-specific lifelong immunity (Yu et al., 2008), humans are routinely re-infected with antigenically drifted influenza strains throughout life. Most humans are infected with seasonal influenza viruses by 3 years of

age (Bodewes et al., 2011) and then re-infected with antigenically drifted strains every 5-10 years (Kucharski et al., 2015).

Influenza infections early in life can leave long-lived immunological imprints. In 1960, Thomas Francis coined the term 'original antigenic sin' to describe the observation that Abs primed by older viral strains often dominate secondary responses elicited by new antigenically drifted viral strains (Francis, 1960). The 'S' in OAS refers to the observation that the recall of Abs generated against past strains can occur at the apparent expense of generating *de novo* Abs that react to new viral strains (de St.Groth and Webster, 1966a; Francis, 1960). It is thought that OAS Abs are detrimental to the host since they react poorly to the viral strain that is actually recalling them.

While it is clear that the majority of human influenza infections take place in the context of a previously exposed host, most animal models do not take prior-exposures into account. Recently, Jacobs and colleagues established a mouse model of OAS (Kim et al., 2012; Kim et al., 2009). These studies show that OAS Abs can be elicited in mice following sequential exposures with antigenically distinct influenza strains. Here, we used a similar mouse model to determine the precise binding footprints and neutralization efficiencies of OAS Abs elicited by sequential influenza exposures. Surprisingly, we found that many OAS Abs target the same general region of HA that is recognized by non-OAS Abs. Further, we found that OAS Abs can be highly effective at neutralizing antigenically distinct viruses *in vivo* and that B cells producing OAS Abs are clonally related to B cells that produce non-OAS Abs. Taken together, our data suggest that OAS Abs are a valuable protective component of secondary influenza immune responses.

Results

Establishment of a mouse model of 'OAS'

We established a mouse model to determine how prior H1N1 influenza A virus exposures influence the generation of new Ab responses against antigenically drifted H1N1 strains. For these studies, we utilized the well-characterized A/Puerto Rico/8/1934 (PR8) and A/Puerto Rico/8/1934-S12a (S12a) H1N1 strains (Caton et al., 1982; Das et al., 2013; Gerhard et al., 1981). Early mAb mapping studies demonstrated that there are 4 dominant antigenic sites on the globular head region of the HA of PR8 (Gerhard et al., 1981). The HAs of PR8 and S12a differ by 13 mutations spread across the 4 dominant HA head antigenic sites (Figure 1A) (Caton et al., 1982; Das et al., 2013). PR8 and S12a viruses are antigenically distinct as determined by HAI assays (Figure 1B-C), which measure Ab inhibition of virus binding to red blood cells. Although the HA stalk region of these viruses is identical (Figure 1A), 98% of PR8-elicited mAbs fail to recognize a virus closely related to the S12a virus in direct binding assays (Das et al., 2013).

We exposed mice to the PR8 virus and then re-exposed the same mice 28 days later with the identical PR8 virus or the antigenically distinct S12a virus. Initial viral exposures were given via the intraperitoneal (i.p.) route and secondary exposures were given via the intravenous (i.v.) route. Secondary exposures were delivered via the i.v. route to enhance our ability to derive hybridomas from sequentially exposed mice.

We first measured PR8-reactive Abs in sera collected from these mice. As expected, PR8-reactive Abs were boosted upon re-exposure with PR8 (Figure 2A). Surprisingly, the antigenically distinct S12a virus was also able to boost PR8-reactive

Abs to levels comparable to those elicited by a homologous PR8-PR8 prime-boost (Figure 2A). This boosting of the PR8 response was not due to non-specific activation of B cell clones initiated by the first viral exposure, since an influenza B strain (B/Lee/1940; unrelated to the PR8 and S12a H1N1 influenza A strains) did not boost PR8 Abs in mice originally exposed to PR8 (Figure 2A). Similar results were obtained when we exposed mice with S12a virus 70 days after the initial PR8 exposure (Figure 3A). Since HAI assays only detect Abs directed against the globular head of HA, we completed additional direct ELISA binding assays. Consistent with our HAI data, PR8-reactive Abs detected by ELISA (which measure head and stalk HA Abs) were similar in sera collected from mice sequentially exposed to PR8 twice and mice sequentially exposed to PR8 and the antigenically distinct S12a virus (Figure 3B).

Classically, OAS describes the process by which B cells stimulated by past viral exposures dominate and suppress the generation of *de novo* activated B cells that recognize new antigens present in drifted viral strains (Francis, 1960). To determine if PR8 pre-exposure prevents the generation of *de novo* Ab responses capable of recognizing S12a, we measured levels of S12a-reactive Abs in sequentially exposed mice. Similar levels of S12a-reactive Abs were present in S12a exposed mice with or without prior PR8 exposure (Figure 2B). Taken together, our data suggest that in PR8-exposed mice, S12a elicits Abs that react to the antigenically distinct PR8 strain but that this does not occur at the expense of eliciting S12a-reactive Abs.

Identification of OAS Abs

Our experiments using sera cannot distinguish whether cross-reactive Abs are preferentially elicited in sequentially exposed mice or if PR8-specific Abs and S12a-

specific Abs are both elicited in sequentially exposed mice. To determine the specificity of Abs elicited in our experimental system, we created a panel of 289 hybridoma cell lines from mice exposed to only PR8, to only S12a, sequentially to PR8 twice, or sequentially to PR8 and S12a (Figure 4). Hybridoma cell lines were created from a total of 23 mice (Figure 5). Consistent with previous studies (Das et al., 2013), a single PR8 exposure elicited mAbs that bound with a high affinity to PR8 but not S12a (Figure 4A). Likewise, the majority of mAbs elicited by a single S12a exposure bound with a high affinity to S12a but not to PR8 (Figure 4B). Most mAbs elicited by PR8-PR8 sequential exposure bound with a high affinity to PR8 and not S12a, although some mAbs elicited under these conditions were able to bind to both viruses (Figure 4C). Sequential exposure with PR8 and S12a elicited a higher proportion of mAbs that were able to bind with a high affinity to both PR8 and S12a (Figure 4D; $p < 0.05$; Fisher's exact test). Almost all of these cross-reactive mAbs bound to PR8 better than to S12a (sample binding curves shown in Figure 6). S12a-specific mAbs were also elicited by PR8-S12a sequential exposure (Figure 4D). Strikingly, approximately half of the mAbs elicited by sequential exposure to PR8 and S12a bound with a high affinity to PR8 and not S12a (Figure 4D). Most of these 'OAS' mAbs bound to S12a so poorly that actual binding affinities to S12a could not be calculated using standard ELISA assays (Figure 7). As a control, we attempted to create hybridoma cell lines from PR8-exposed mice that were subsequently exposed to an unrelated influenza B strain. We were unable to isolate PR8-reactive mAbs under these conditions, which indicates that some level of antigenic-relatedness is required to recall PR8-reactive Abs in PR8 pre-exposed mice.

Specificity and functionality of OAS Abs

We mapped the binding footprints of each mAb by measuring binding to a large panel of PR8 viruses that possessed single mutations in the HA (Figure 8). Our mutant PR8 viral panel included previously characterized mAb escape mutants (Caton et al., 1982), as well as new escape mutants that we isolated after incubating PR8 virus with mAbs from this study (for example, the K189N HA mutant was isolated after growing PR8 virus in the presence of the H5-60A mAb). We tested mAb binding to viruses that possessed single mutations across the 4 dominant HA antigenic sites (Sa, Sb, Ca, and Cb). The majority of mAbs isolated from mice sequentially exposed to PR8-PR8 and PR8-S12a did not bind to PR8 viruses possessing Sb mutations (Figure 8A-B, Figure 9), indicating that both PR8-PR8 and PR8-S12a sequential exposures elicit an Ab response that is immunodominant against epitopes in the Sb antigenic site of HA. Approximately ½ of the Sb mAbs isolated from mice sequentially exposed to PR8 and S12a displayed a classical OAS phenotype since they bound poorly to S12a (Figure 8B). Importantly, the other ~ ½ of the Sb mAbs isolated from mice sequentially exposed to PR8 and S12a were cross-reactive with strong binding to both PR8 and S12a (Figure 8B). mAbs isolated from mice receiving only a single PR8 or S12a exposure reacted to all 4 HA globular head antigenic sites and were not as frequently directed against the Sb antigenic site (Figure 9), indicating that sequential viral exposure focused the Ab response towards the Sb antigenic site.

We next sought to determine if there are fine specificity differences that distinguish Sb mAbs isolated from mice exposed with PR8-PR8 compared to PR8-S12a. To do this, we completed additional mAb binding experiments with a larger panel of PR8 viruses with single Sb mutations (Figure 10). There was a notable key difference in the

binding footprint of Sb mAbs isolated from mice sequentially exposed to PR8-PR8 compared to PR8-S12a: the majority (70%) of Sb mAbs isolated from mice sequentially exposed twice with PR8 failed to bind to viruses with a mutation at HA residue 156, whereas the minority (20%) of Sb mAbs isolated from mice sequentially exposed with PR8 and S12a were sensitive to the 156 mutation (Figure 11A-B and Figure 10). This is important because the Sb antigenic site of PR8 and S12a differ at residue 156 (PR8 has E156 and S12a has K156). Most of the 'OAS' Sb Abs that were elicited by PR8-S12a sequential exposure did not bind to PR8 viruses with an E156K HA mutation (these Abs had a similar footprint to most Sb Abs elicited by PR8-PR8 sequential exposure), whereas most of the cross-reactive Sb Abs elicited by PR8-S12a sequential exposure were less affected by the E156K mutation (Figure 8B and Figure 10).

To determine if mAbs isolates from mice sequentially exposed to PR8 and S12a can protect against viral infection, we completed a series of passive transfer studies in mice. We transferred mAbs that were either sensitive (H2-S37B) or resistant (H4-S10B) to the E156K HA mutation and we then challenged mice with either PR8 or S12a virus. Both mAbs protected mice against PR8 infection (Figure 11C). The H2-S37B mAb that was sensitive to the PR8 E156K HA mutation failed to protect mice against the S12a virus, however the H4-S10B mAb that was resistant to the PR8 E156K HA mutation fully protected mice against the S12a virus (Figure 11D). The E156K-resistant mAb was able to protect against S12a virus infection, despite binding to S12a with a much lower affinity compared to PR8 (Figure 8B).

These data suggest that sequential exposure with PR8 and S12a elicit two closely related but clearly distinct types of Sb-specific Abs. The first type of Ab displays a classical OAS phenotype in that these Abs have minimal cross-reactivity to S12a or a

PR8 virus engineered to have the E156K HA mutation, which is the mutation that is largely responsible for the Sb antigenic difference between PR8 and S12a. The second type of Ab targets the same general Sb region but binds strongly to both PR8 and S12a and is not sensitive to variation at residue 156. These cross-reactive Abs appear to have a narrower binding footprint based on our binding assays using a large panel of Sb mutant PR8 viruses (Figure 10).

Somatic mutations affect the cross-reactivity of Sb mAbs

In an attempt to identify clonally related mAbs, we sequenced the heavy and light chains of a number of our hybridoma cell lines. We identified a group of 6 clonally related IgG2a mAbs derived from a single mouse that was sequentially exposed to PR8 and S12a (Table 1). These 6 related mAbs had different binding affinities to PR8 and S12a (Figure 12). For example, the H5-42B mAb had very weak binding to S12a (Figure 12A), whereas the H5-61B mAb, which differed by only 4 amino acids in the heavy chain relative to H5-42B (1 CDR1, 2 FR3, and 1 CDR3 differences), had dramatically increased binding to S12a (Figure 12B). The H5-61B mAb bound better to a PR8 mutant virus possessing the E156K Sb mutation compared to the H5-42B mAb (Figure 8). It is unclear if the mutations that differentiate H5-42B and H5-61B originated during the initial priming with the PR8 virus or during the secondary recall response against S12a. Regardless, these data suggest that somatic mutations can influence the level of cross-reactivity of Abs elicited by secondary influenza exposures.

To determine if the H5-42B and H5-61B mAbs have different protective efficiencies, we completed *in vivo* passive transfer experiments. As an added control, we measured HAI titers of sera collected 12 hours after mAb transfer at the time of

infection. Ab titers were not significantly different between mice receiving the H5-42B or H5-61B mAbs, and the average HAI titer against PR8 was 50, which is well below the average HAI titers of 140 elicited after a primary response to immunization with 1000 HAU PR8 (Figure 13C). Both the H5-42B and H5-61B mAbs inhibited PR8 infection following passive transfer *in vivo* (Figure 13A). Surprisingly, despite the observation that H5-42B binds poorly to S12a (Figure 8), both mAbs also inhibited S12a infection following passive transfer *in vivo* (Figure 13B). The H5-61B mAb, which had a higher relative binding affinity to S12a compared to H5-42B, was more protective following S12a infection *in vivo* compared to H5-42B (Figure 13B). As a comparison, we also tested mAbs isolated from mice following a primary PR8 exposure (the H0-P2D5D10 mAb) or a primary S12a exposure (the H7-S2-2A mAb). As expected, the PR8 primary mAb protected mice only against PR8 infection and the S12a primary mAb protected mice only against S12a infection (Figure 13A-B). Importantly, the S12a primary mAb protected mice against S12a infection to the same extent as the H5-42B mAb elicited by PR8-S12a sequential exposure. These data indicate that Abs elicited by sequential exposures can be highly effective at neutralizing the virus that recalls them, even though they often bind with a much higher affinity to the first viral strain that the host encountered. These Abs can be as effective as Abs elicited in a previously naïve host.

Discussion

Most vaccines elicit protective Abs and MBCs that are poised to respond quickly to a secondary exposure (Plotkin, 2010). Formulating effective vaccines against rapidly changing pathogens, such as influenza viruses, is challenging (Hensley, 2014; Schultz-Cherry and Jones, 2010; Stohr et al., 2012). Influenza vaccines are less effective when viruses acquire mutations in Ab binding sites on the HA, as was the case during the

2014-2015 influenza season (Chambers et al., 2015). B cells primed against older influenza strains are recruited upon exposure with new influenza strains, even when major HA antigenic changes have occurred. For example, prior seasonal H1N1 (sH1N1) exposure heavily influenced the types of Abs that were elicited in humans against the 2009 pandemic H1N1 strain (Li et al., 2012; Li et al., 2013; Linderman et al., 2014; Wrammert et al., 2011). mAbs isolated from humans exposed to the 2009 pH1N1 recognize epitopes that are conserved on older sH1N1 strains, and these mAbs have a high level of somatic mutation indicating that they were likely derived from a memory B cell pool (Wrammert et al., 2011). Immune skewing towards epitopes present in older viral strains is likely a result of MBC clones outcompeting naïve B cell clones that are specific for epitopes present in new viral strains (Engels et al., 2009; Kometani et al., 2013; Liu et al., 2012). In the case of the 2009 pH1N1 strain, many of these cross-reactive mAbs were protective and so the recall of B cell clones originally primed by sH1N1 viruses was likely advantageous to the host.

There is evidence that prior influenza exposures can negatively affect priming of Ab responses against new viral strains through a process commonly called OAS (Davenport et al., 1953; Fazekas de St and Webster, 1966; Francis, 1955; Francis, 1960; Webster, 1966). Prior influenza exposures can decrease the generation of *de novo* Ab responses, but this is partially due to cross-reactive immunity (originally elicited by prior exposures) eliminating antigen following secondary exposure with antigenically drifted strains (Kim et al., 2009). New Ab responses are not primed effectively when cross-reactive Abs limit antigen levels. In this context, prior immunity is again advantageous because Abs elicited against prior influenza exposures are partially

protective and limit the amount of virus and antigen expressed upon secondary exposure.

A poorly understood component of OAS is that antigenically distinct viruses recall some Abs that paradoxically only react to older viral strains and not to the viral strain that is actually eliciting the response. Our data indicate that there are surprisingly only subtle differences between OAS and cross-reactive Abs. We expected that sequential exposure with the PR8 and S12a strains would elicit Ab responses directed towards the HA stalk, since this region of HA is fully conserved between the two viral strains. However, we found that the majority of Abs elicited by PR8-S12a sequential exposure were directed against the Sb antigenic site of HA, which differs significantly between the two viral strains. Some Sb-specific mAbs elicited by sequential exposure exhibited a classical OAS phenotype in that they bound very poorly to S12a but bound with a high affinity to PR8. Other Sb-specific mAbs elicited in the same mice were more cross-reactive and bound to both PR8 and S12a. The most important part of our study is that we discovered that both OAS and cross-reactive Sb Abs recognized the same epitope within the Sb antigenic site and that cross-reactive Sb Abs were more tolerant of antigenic differences within this epitope. Very few somatic mutations were required to increase S12a-reactivity of OAS Abs, indicating that OAS and cross-reactive Abs can be clonally related.

Although OAS Abs are typically thought of as detrimental, OAS Abs elicited in our experiments were able to protect mice from S12a infections in passive mAb transfer experiments. This, along with the observation that B cells producing OAS Abs are potentially precursors for B cells that produce more cross-reactive Abs, suggests that

OAS Abs play an important role in protecting the host against secondary encounters with antigenically drifted viral strains.

Materials and Methods

Animal Experiments

All murine experiments were performed at the Wistar Institute according to protocols approved by the Wistar Institute Institutional Animal Care and Use Committee. All mice were BALB/c strain female mice of at least 7 weeks of age.

Viruses

Viruses were grown in 10-day old fertilized chicken eggs and the HA genes of each virus stock were sequenced to verify that additional mutations did not arise during propagation. S12a has an additional K144E HA mutation compared to the previously published Seq12 virus (Das et al., 2013). Seq12 viruses possessing the K144E HA mutation grow efficiently in fertilized chicken eggs without acquiring additional HA mutations. Some virus mutants were generated by reverse genetics as previously described (Martínez-Sobrido and García-Sastre, 2010). Selected mutations were added to the HA gene in a pDZ plasmid using a QuikChange II XL site-directed mutagenesis kit (Agilent). As controls, we used B/Lee/1940 and the A/Puerto Rico/08/1934-J1 strain that has an H3 HA from A/Hong Kong/01/1968.

HAU Assays

To determine the hemagglutination unit (HAU) titer of each viral prep, viruses were diluted 2-fold across a 96-well round bottom plate (BD) and mixed with 12.5ul of

2% (vol/vol) turkey erythrocytes (Lampire) in phosphate-buffered saline (PBS) (Cellgro). Agglutination was read out after incubating for 60 minutes at room temperature.

50% Tissue Culture Infectious Dose (TCID50) Assays

96-well flat bottom plates (BD) were incubated overnight with 4e4 Madin Darby canine kidney (MDCK) cells/well in minimum essential medium (MEM) (Cellgro) with 9% fetal bovine serum (FBS) (Sigma). Plates were washed 3 times with serum-free MEM. Viruses were diluted 10-fold across the plate in MEM supplemented with 0.1% gentamicin (Gibco), 1ug/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington), and 5 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (Cellgro).

HAI Assays

Sera were collected from mice by submandibular bleeding into 1.1ml Serum Gel Z microtubes (Sarstedt) using a 5 mm lancet (Medipoint). Sera were then heat treated for 30 minutes at 55C. HAI titrations were performed in 96-well round bottom plates. Sera were serially diluted 2-fold in PBS and added to 4 agglutinating doses of virus in a total volume of 100 µL. Turkey erythrocytes were added [12.5 µL of a 2% (vol/vol) solution]. The erythrocytes were gently mixed with sera and virus and agglutination was read out after incubating for 60 min at room temperature. HAI titers are expressed as the inverse of the highest dilution that inhibited 4 agglutinating doses of virus.

ELISA

Viruses for ELISAs were inactivated by B-Propiolactone (BPL; Sigma-Aldrich) treatment. Viruses were incubated with 0.1% BPL and 0.1 M HEPES overnight at 4C

followed by a 90 minute incubation at 37C. 96-well Immulon 4HBX flat-bottom microtiter plates (Fisher Scientific) were coated with 20 HAU per well BPL-treated virus overnight at 4C. Plates were then incubated with 150ul 3% bovine serum albumin (BSA) (Sigma) in PBS for 2 hours at room temperature. For initial hybridoma screening, hybridoma supernatants were diluted 1:5 and then added to ELISA plates. For Ab footprint mapping, purified mAbs were added at a starting concentration of 1 to 10 ug/ml and diluted 2-fold across the plate. As a positive control, we used the stalk-reactive C179 mAb (Clontech). As a negative control and to assure that mAbs bound to HA, we used the J1 virus strain which has an H3 HA. mAbs were incubated on the plates for 2 hours at room temperature. mAb binding was measured by a secondary goat anti mouse IgG or IgM Ab conjugated to alkaline phosphatase (Southern Biotech) incubated for 1 hour at room temperature. Plates were developed with 100ul/well of a 2.7mM p-nitrophenyl phosphate (PNPP) (Thermo Scientific) 0.1M $NaHCO_3$ (Sigma-Aldrich) 1mM $MgCl_2$ (Fisher Scientific) pH 9.8 solution. Absorbance was measured at 405nm. Plates were washed with distilled water between each incubation step.

Hybridoma production

Some mice were first primed with 1000 HAU of virus with an i.p. injection and boosted 28 days later with 1000 HAU of virus with an i.v. injection. 3 days later, splenocytes from these mice were fused with SP2/0 cells using Polyethylene glycol (Hybrimax 50% (w/v) Sigma-Aldrich). Some mice were primed only once i.v. with 1000 HAU of virus and splenocytes from these mice were fused 5 days later. Fused cells were incubated in 5.7uM azaserine and 100uM hypoxanthine (Azaserine-hypoxanthine Sigma-Aldrich) with 10% FBS in Dulbecco's modified eagle medium (DMEM) (Cellgro). 10 days later hybridomas were screened by ELISA as described above. Positive clones

were subcloned by limiting dilution, and then grown in DMEM supplemented with Zap hybridoma (InVitria). mAbs were purified using PureProteome A/G coated magnetic beads (Millipore). For mAb transfer experiments, mAbs underwent buffer exchange into PBS using Zeba desalt spin columns (Thermo Scientific).

mAb transfer experiments

25 ug of purified mAbs diluted in PBS were transferred into female BALB/c mice (Charles River Laboratories) in a 200ul i.p injection. 12 hours later, mice were challenged intranasally with a dose of 30 TCID50 of either PR8 or S12a in 50ul PBS. For intranasal infections, mice were first anesthetized by inhalation of isoflurane (Henry Schein). Weight was measured daily for 14 days.

Immunoglobulin sequencing

DNA was isolated from hybridoma clones using a Gentra Puregene cell kit (Qiagen). Immunoglobulin heavy and light chain genes were amplified by PCR using a Platinum Taq DNA Polymerase High Fidelity kit (Thermo Fisher Scientific) using previously described primers (Ramsden et al., 1994; Wang et al., 2000). Amplified DNA was run on a 1% agarose (Lonza) in TAE buffer (Corning) gel and DNA was purified from the gel using a Zymoclean gel DNA recovery kit (Zymo Research). DNA fragments were then sequenced by Sanger sequencing. For hybridomas whose productive and non-productive rearrangements were not easily separated by gel electrophoresis, the DNA was subcloned using a TOPO TA Cloning kit with the pCRII-TOPO vector (Invitrogen).

mAb selection assay

To select for viral escape mutants to selected mAbs, 96 well flat bottom plates were coated with 4e4 MDCK cells/well in MEM with 9% FBS and incubated overnight at 37C. Virus used in this assay had been serially passaged in eggs a minimum of three times to increase mutant frequency. Virus was incubated with four different concentrations of virus to determine a saturating concentration of mAb for one hour at room temperature. MDCK cell-coated plates were washed four times with serum free MEM and 100ul of the virus/mAb mixture were transferred to the cells and incubated for two hours at 37C. Plates were subsequently washed and coated with 100ul of MEM with 0.1% gentamicin, 1ug/ml TPCK-treated trypsin, and 5 mM HEPES supplemented with mAb. Plates were incubated for three days at 37C. At this time, cytopathology was determined and virus collected from the infection endpoints of those wells that had a saturating mAb concentration. vRNA was collected using a QIAamp viral RNA kit (Qiagen) and sequenced to determine the mutation that enabled escape of mAb mediated neutralization. Virus escape mutants collected were subsequently generated by reverse genetics, or were subcloned by viral plaque assay. As a control, cells were incubated with virus but without mAb to determine the mutant frequency in the viral population.

Viral plaque assay

To subclone virus, 6 well plates (BD) were coated with 5e5 MDCK cells/well in MEM with 9% FBS and incubated overnight at 37C. Plates were washed four times with MEM and coated with 500ul serially diluted virus in MEM. Plates were incubated at 37C for 1 hour with plates rocked every 15 minutes. Plates were washed with MEM and coated with 1.6% agarose in MEM with 10mM HEPES, 0.1% gentamicin, 1ug/ml TPCK-

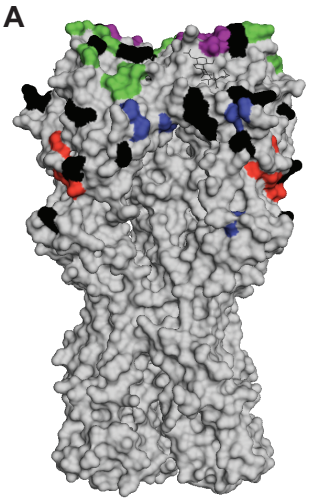
treated trypsin, and 0.05% phenol red. Three days post infection, isolated plaques were collected, confirmed by sequencing, and grown in 10 day old fertilized chicken eggs.

Statistical analyses

Student's t tests, Fisher's exact tests, area under the curve analyses, standard deviation (SD), and standard error of the mean (SEM) were calculated using Graphpad Prism6 software (Graphpad Software Inc).

Figures and Table

Figure 1



Antigenic Site	Mutations between PR8 and S12a
Sa	N129D, K163E
Sb	E156K, G159S
Ca	E70G, K144E, S145N, G173R, R224K, D225G
Cb	R48K, R78G, E119K

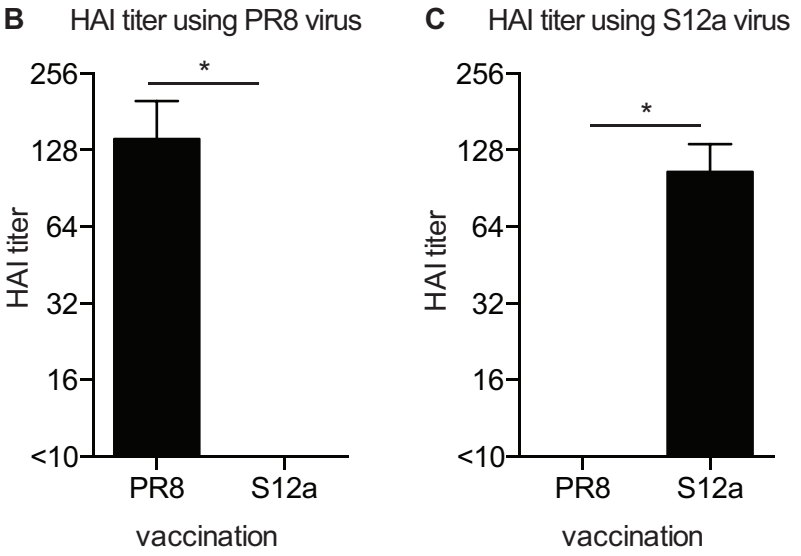


Figure 1. PR8 and S12a are antigenically distinct H1N1 viruses.

(A) PR8 and S12a differ at 13 HA residues (highlighted in black) (Protein database: 1RVX). The Sa, Sb, Ca, and Cb antigenic sites are shown in green, purple, blue, and red respectively. (B,C) Mice were immunized i.p. with 1000 HAU PR8 or S12a virus.

Sera were collected 21 days post-vaccination and HAI assays were completed using PR8 (B) or S12a (C) virus. Data are mean +/- SD (*$p < 0.0001$; Student's t test).

Figure 2

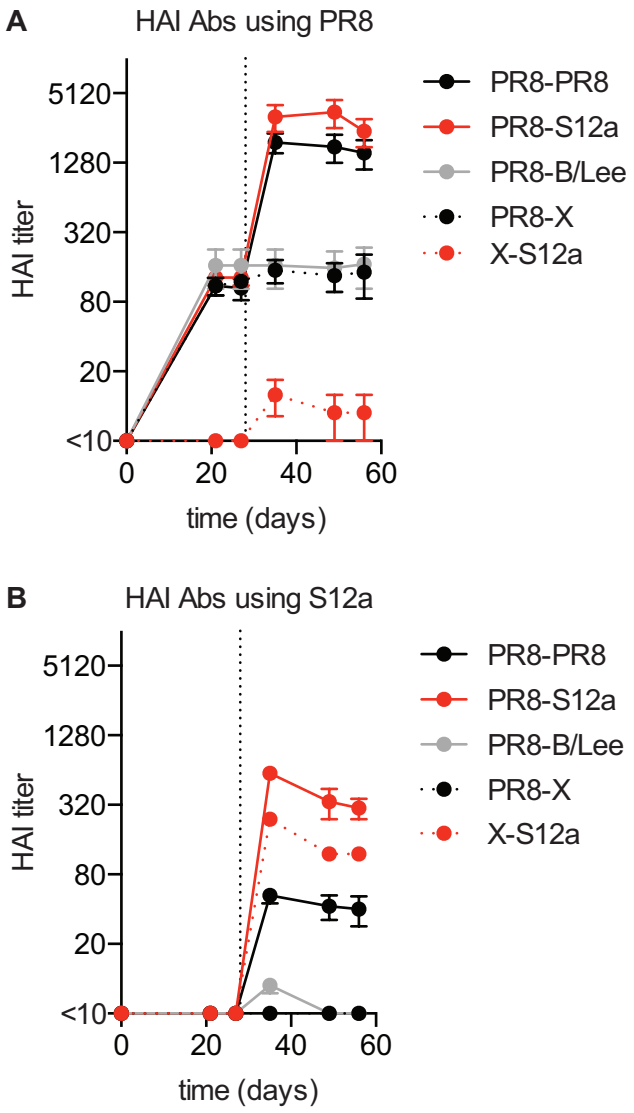


Figure 2. S12a efficiently boosts Abs that react to PR8 HA.

(A,B) Seven week old BALB/c mice were immunized i.p. with 1000 HAU of PR8 or PBS (X) and boosted 28 days later i.v. with 1000 HAU PR8, 1000 HAU S12a, 1000 HAU B/Lee, or PBS (X) (n = 4 mice per group). Sera were collected at different times after immunization and HAI assays were completed using PR8 (A) and S12a (B) viruses. Data are mean +/- SEM. Mice boosted with both PR8 or S12a had significantly higher

PR8 HAI titers compared with mice boosted with PBS (X) or the antigenically unrelated B/Lee virus ($p < 0.05$; Student's t test).

Figure 3

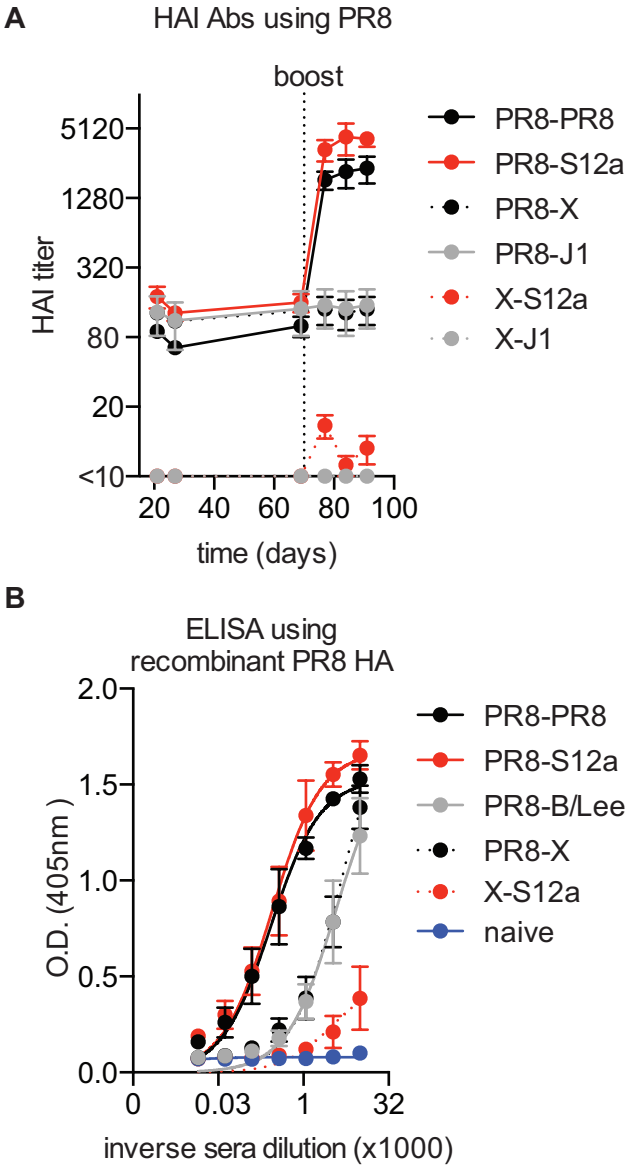


Figure 3. S12a boosts PR8 Abs at late time points after initial exposure.

(A) Mice were immunized i.p. with 1000 HAU of PR8 or PBS (X). 70 days later, mice were boosted i.v. with 1000 HAU PR8, S12a, J1, or PBS (X). Sera were collected at different times after immunization and HAI assays were completed using PR8 virus. Mice boosted with PR8 or S12a had significantly higher PR8 HAI titers compared to mice boosted with PBS or the unrelated J1 for at least 3 weeks post-boost ($p < 0.05$;

Student's t test). (B) Mice were immunized i.p. with 1000 HAU PR8 or PBS (X). Mice were boosted i.v. with 1000 HAU PR8, S12a, or B/Lee 28 days later. Sera were collected 21 days after the boost and tested by ELISA against PR8 virus. Sera from mice that were boosted with PR8 or S12a had significantly higher PR8 titers than mice that were boosted with negative controls PBS or B/Lee ($p < 0.05$; Student's t test of area under the curve).

Figure 4

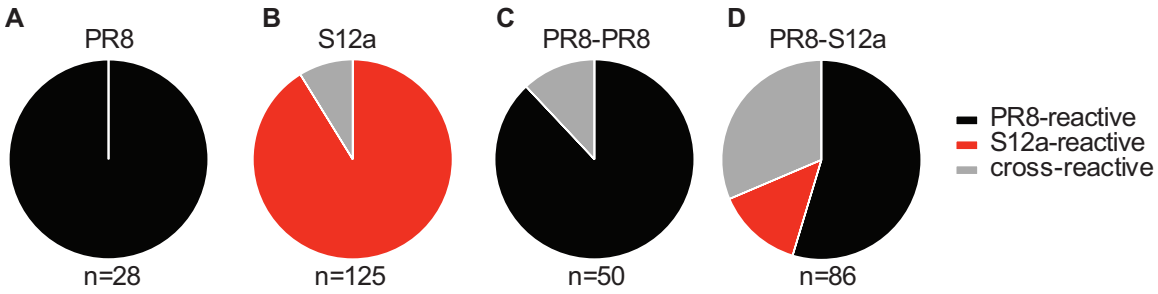


Figure 4. PR8-S12a sequential exposure elicits many Abs with a classical OAS phenotype.

(A, B) Mice were immunized i.v. with 1000 HAU of PR8 (A) or S12a (B) virus. Hybridomas were created from splenocytes collected 5 days after vaccination. (C, D) Mice were immunized i.p. with 1000 HAU of PR8 and boosted i.v 28 days later with 1000 HAU PR8 (C) or S12a (D). Hybridomas were produced 3 days after the boost. We completed ELISAs with a range of different mAb concentrations using plates coated with PR8, S12a, and J1 (J1 is a virus that has an H3 HA). mAbs were determined to be PR8 and/or S12a-specific if they produced an ELISA signal that was 4 times greater using PR8 or S12a coated plates compared to plates coated with the J1 negative control. Mice boosted with S12a had a higher proportion of Abs cross-reactive for both PR8 and S12a ($p < 0.005$; Fisher's exact test).

Figure 5

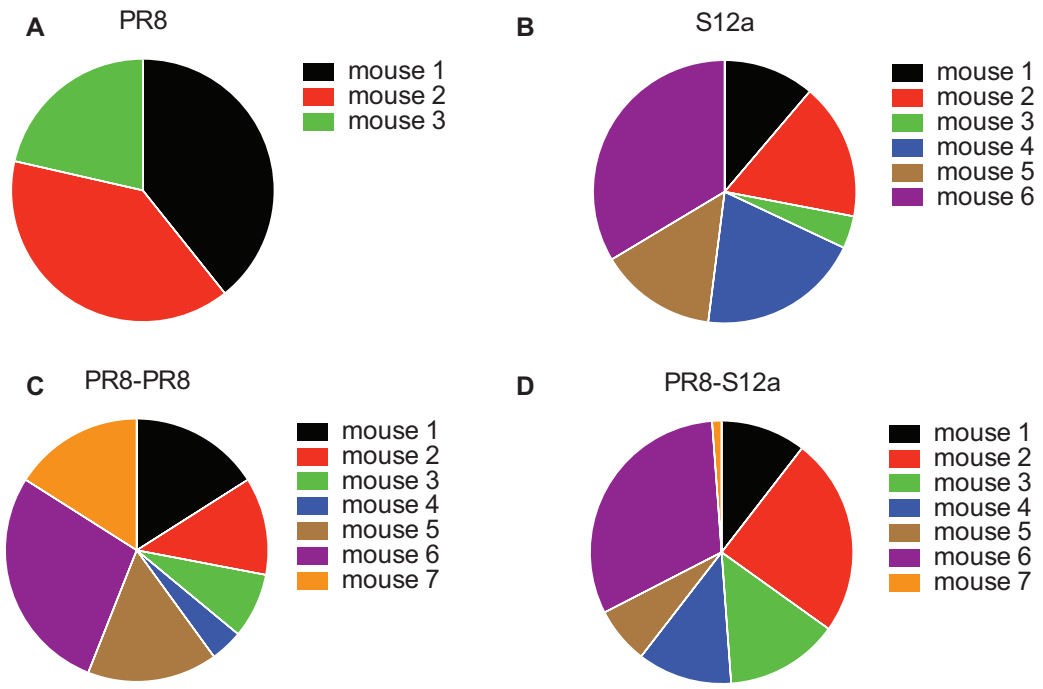


Figure 5. Hybridomas for these experiments were derived from many mice.

Shown are the fraction of mAbs that were derived from individual mice. (A) 28 mAbs were isolated from mice exposed to PR8. (B) 125 mAbs were isolated from mice exposed to S12a. (C) 50 mAbs were isolated from mice sequentially exposed to PR8 twice. (D) 86 mAbs were isolated from mice sequentially exposed to PR8 and S12a. We did not use many mice for PR8 primary exposure since previous studies have characterized mAbs isolated from mice following a single PR8 exposure.

Figure 6

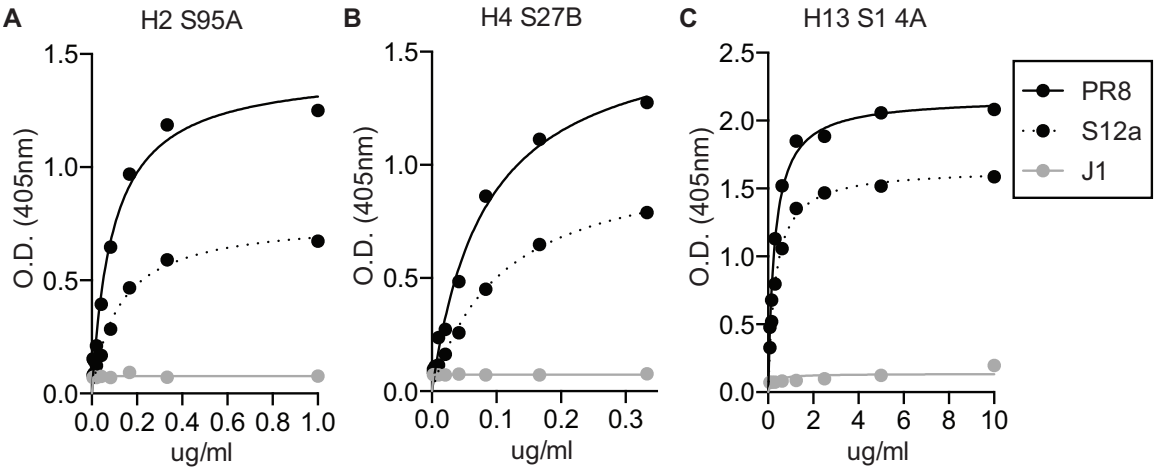


Figure 6. Most 'cross-reactive' mAbs bind better to PR8 than to S12a.

(A-C) mAbs were tested by ELISA for reactivity against PR8, S12a, and J1 virus. Shown are 3 mAbs that bound with a higher relative affinity to PR8 than to S12a. As an ELISA coating control, we used the stalk-specific C179 mAb that bound with similar affinities to both PR8 and S12a (not shown).

Figure 7

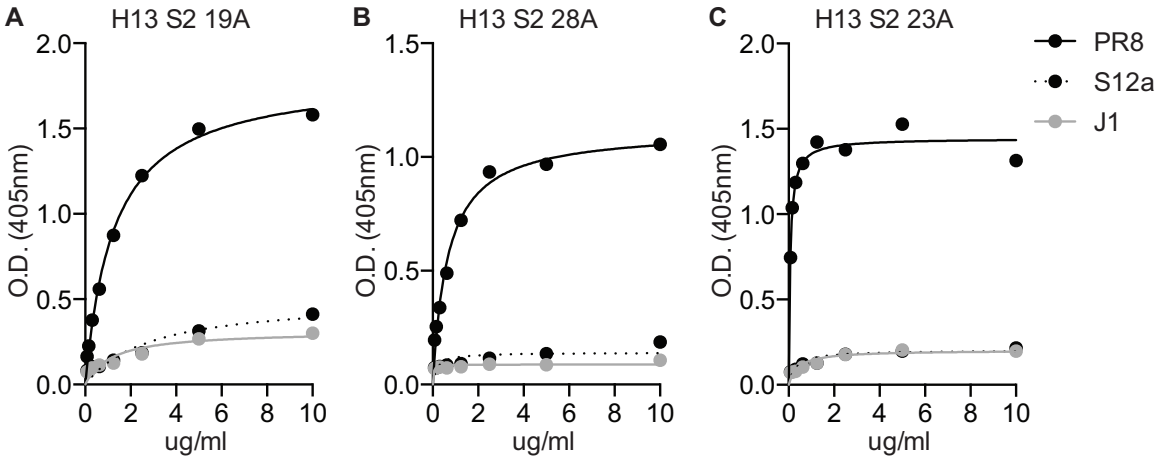


Figure 7. 'OAS' mAbs bind very poorly to S12a.

(A-C) mAbs were tested by ELISA for reactivity against PR8, S12a, and J1 virus. Shown are three mAbs elicited in mice that received a PR8-S12a prime-boost vaccination that had measurable binding to PR8 but not to S12a. As an ELISA coating control, we used the stalk-specific C179 mAb that bound with similar affinities to both PR8 and S12a (not shown).

Figure 8

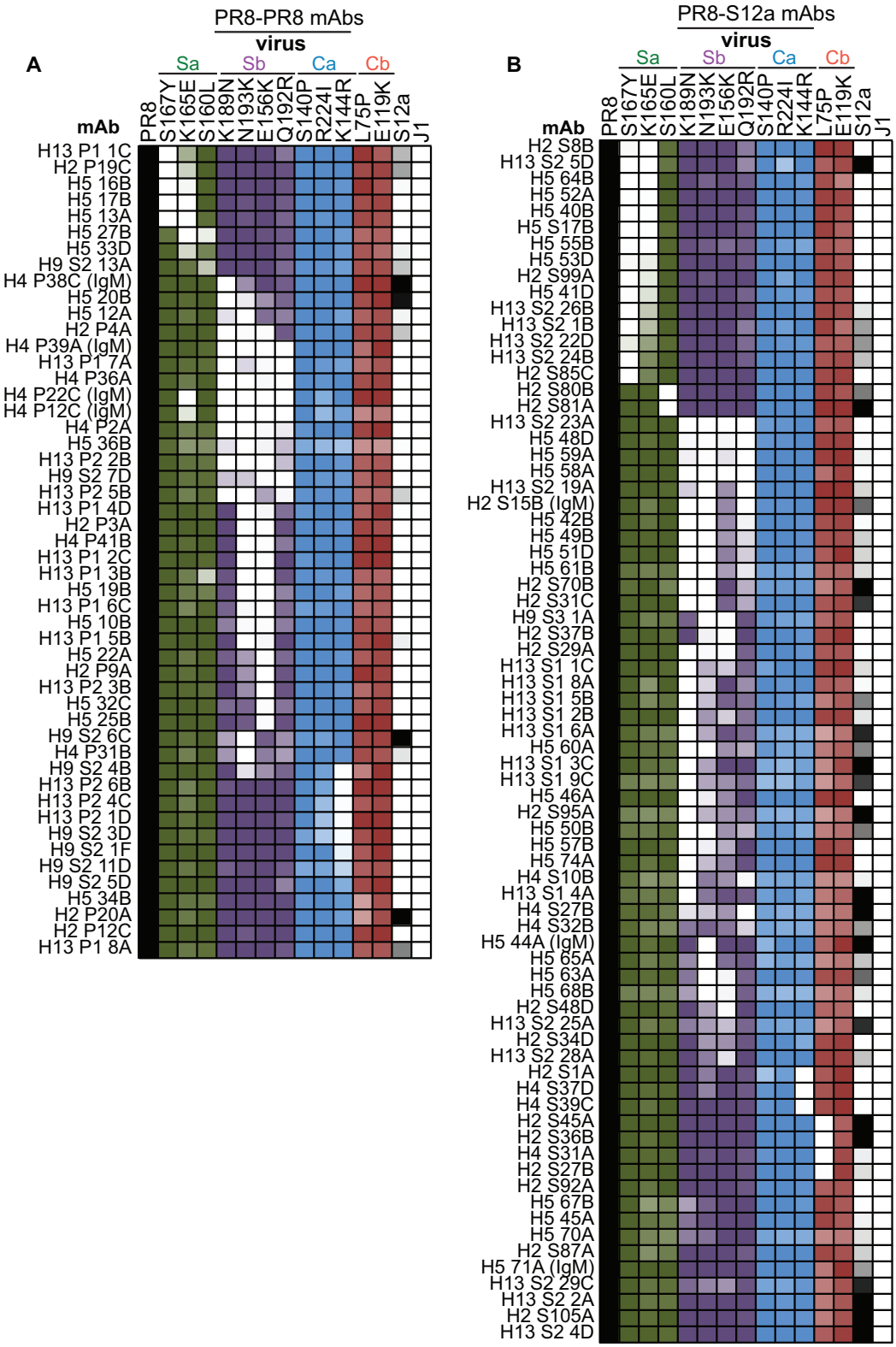

A
PR8-PR8 mAbs
virus
Sa
Sb
Ca
Cb
mAb
PR8
S167Y
K165E
S160L
K189N
N193K
E156K
Q192R
S140P
R224I
K144R
L75P
E119K
S12a
J1
H13 P1 1C
H2 P19C
H5 16B
H5 17B
H5 13A
H5 27B
H5 33D
H9 S2 13A
H4 P38C (IgM)
H5 20B
H5 12A
H2 P4A
H4 P39A (IgM)
H13 P1 7A
H4 P36A
H4 P22C (IgM)
H4 P12C (IgM)
H4 P2A
H5 36B
H13 P2 2B
H9 S2 7D
H13 P2 5B
H13 P1 4D
H2 P3A
H4 P41B
H13 P1 2C
H13 P1 3B
H5 19B
H13 P1 6C
H5 10B
H13 P1 5B
H5 22A
H2 P9A
H13 P2 3B
H5 32C
H5 25B
H9 S2 6C
H4 P31B
H9 S2 4B
H13 P2 6B
H13 P2 4C
H13 P2 1D
H9 S2 3D
H9 S2 1F
H9 S2 11D
H9 S2 5D
H5 34B
H2 P20A
H2 P12C
H13 P1 8A
B
PR8-S12a mAbs
virus
Sa
Sb
Ca
Cb
mAb
PR8
S167Y
K165E
S160L
K189N
N193K
E156K
Q192R
S140P
R224I
K144R
L75P
E119K
S12a
J1
H2 S8B
H13 S2 5D
H5 64B
H5 52A
H5 40B
H5 S17B
H5 55B
H5 53D
H2 S99A
H5 41D
H13 S2 26B
H13 S2 1B
H13 S2 22D
H13 S2 24B
H2 S85C
H2 S80B
H2 S81A
H13 S2 23A
H5 48D
H5 59A
H5 58A
H13 S2 19A
H2 S15B (IgM)
H5 42B
H5 49B
H5 51D
H5 61B
H2 S70B
H2 S31C
H9 S3 1A
H2 S37B
H2 S29A
H13 S1 1C
H13 S1 8A
H13 S1 5B
H13 S1 2B
H13 S1 6A
H5 60A
H13 S1 3C
H13 S1 9C
H5 46A
H2 S95A
H5 50B
H5 57B
H5 74A
H4 S10B
H13 S1 4A
H4 S27B
H4 S32B
H5 44A (IgM)
H5 65A
H5 63A
H5 68B
H2 S48D
H13 S2 25A
H2 S34D
H13 S2 28A
H2 S1A
H4 S37D
H4 S39C
H2 S45A
H2 S36B
H4 S31A
H2 S27B
H2 S92A
H5 67B
H5 45A
H5 70A
H2 S87A
H5 71A (IgM)
H13 S2 29C
H13 S2 2A
H2 S105A
H13 S2 4D

Figure 8. Fine specificity of mAbs elicited by sequential H1N1 exposure.

(A, B) mAbs created from PR8 pre-exposed mice that were boosted either with PR8 (A) or S12a (B) were tested for binding to a panel of viruses by ELISA. We first completed a dose titration of each mAb on ELISA plates coated with PR8. We determined the lowest concentration of each mAb at which PR8 ELISA signal was within 90% of Bmax, and we tested this mAb concentration against all of the mutant viruses. The shade of color in each square is relative to the percentage of binding to that virus compared to PR8 binding with the darkest color being 100% and white being 0%. The coloring scheme (Sa=green, Sb=purple, Ca=blue, Cb=red) matches the coloring scheme of the HA structure shown in Figure 1.

Figure 9

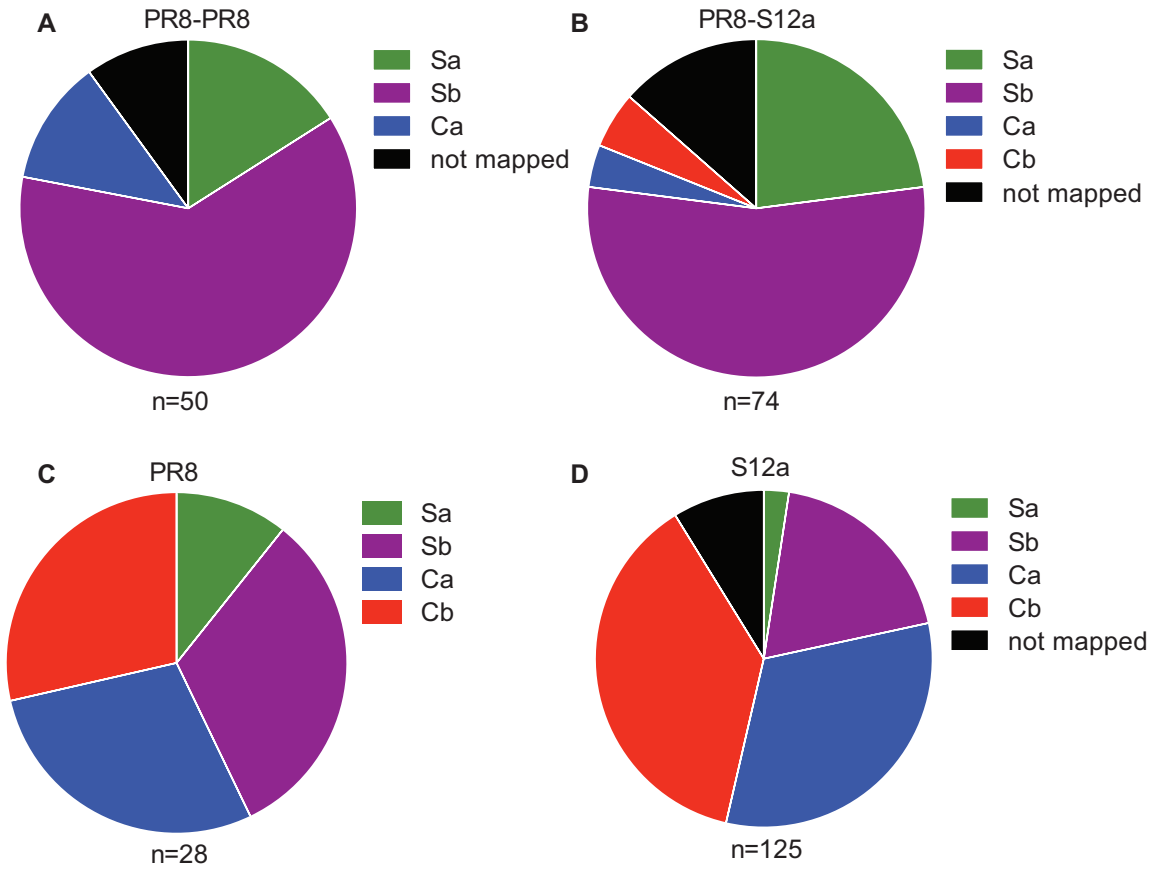


Figure 9. The HA Ab response is focused on the Sb antigenic site following sequential exposure.

(A-B) Hybridomas derived from mice that were vaccinated with PR8 virus and boosted 28 days later with either PR8 (A) or S12a (B) were predominantly specific for the Sb antigenic site. (C-D) This was in contrast to hybridomas derived from mice that were immunized only with PR8 (C) or S12a (D) ($p < 0.05$; Fisher's exact test).

Figure 10

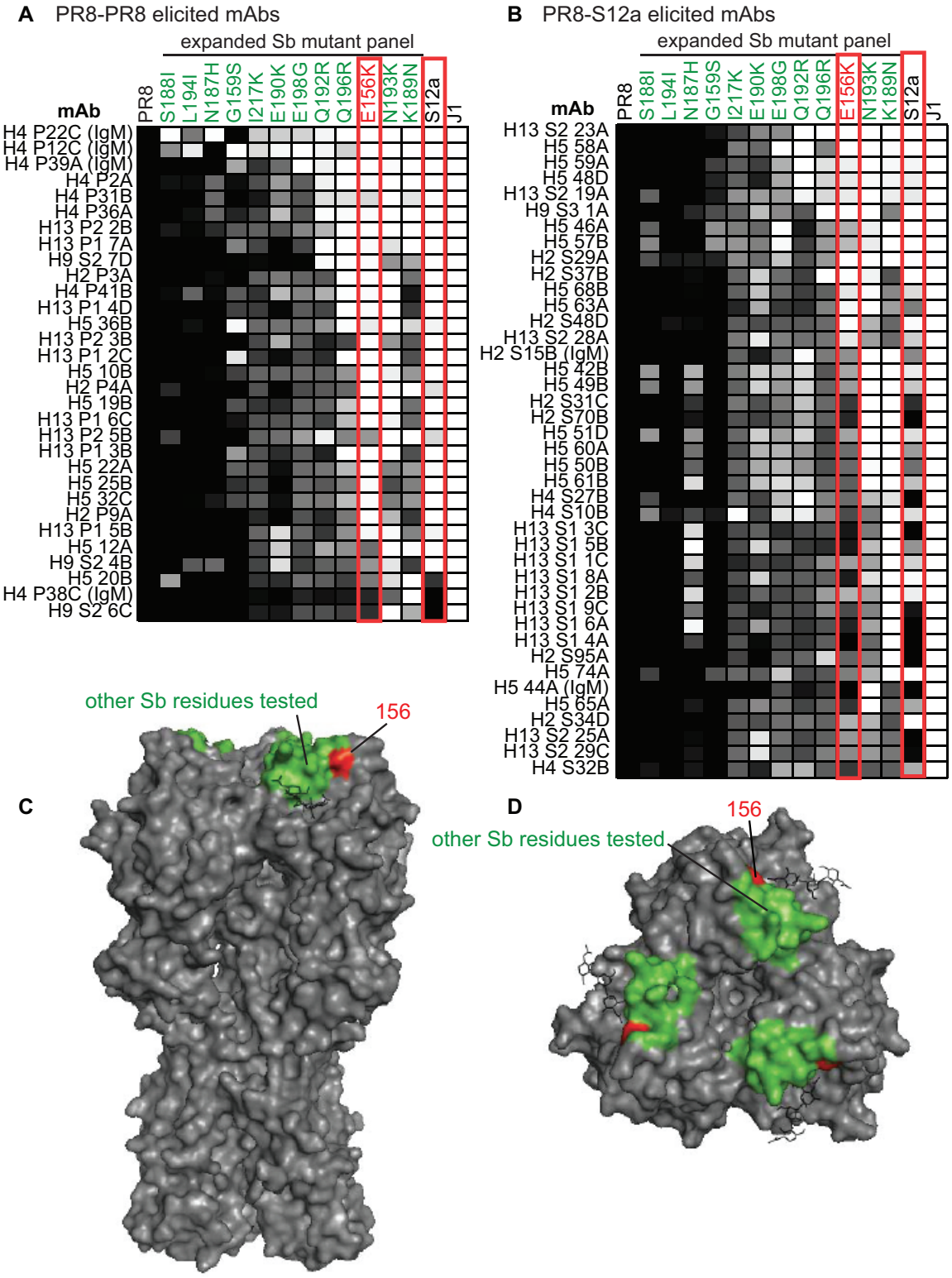

A PR8-PR8 elicited mAbs
expanded Sb mutant panel
mAb
PR8
S188I
L194I
N187H
G159S
I217K
E190K
E198G
Q192R
Q196R
E156K
N193K
K189N
S12a
J1
H4 P22C (IgM)
H4 P12C (IgM)
H4 P39A (IgM)
H4 P2A
H4 P31B
H4 P36A
H13 P2 2B
H13 P1 7A
H9 S2 7D
H2 P3A
H4 P41B
H13 P1 4D
H5 36B
H13 P2 3B
H13 P1 2C
H5 10B
H2 P4A
H5 19B
H13 P1 6C
H13 P2 5B
H13 P1 3B
H5 22A
H5 25B
H5 32C
H2 P9A
H13 P1 5B
H5 12A
H9 S2 4B
H5 20B
H4 P38C (IgM)
H9 S2 6C
B PR8-S12a elicited mAbs
expanded Sb mutant panel
mAb
PR8
S188I
L194I
N187H
G159S
I217K
E190K
E198G
Q192R
Q196R
E156K
N193K
K189N
S12a
J1
H13 S2 23A
H5 58A
H5 59A
H5 48D
H13 S2 19A
H9 S3 1A
H5 46A
H5 57B
H2 S29A
H2 S37B
H5 68B
H5 63A
H2 S48D
H13 S2 28A
H2 S15B (IgM)
H5 42B
H5 49B
H2 S31C
H2 S70B
H5 51D
H5 60A
H5 50B
H5 61B
H4 S27B
H4 S10B
H13 S1 3C
H13 S1 5B
H13 S1 1C
H13 S1 8A
H13 S1 2B
H13 S1 9C
H13 S1 6A
H13 S1 4A
H2 S95A
H5 74A
H5 44A (IgM)
H5 65A
H2 S34D
H13 S2 25A
H13 S2 29C
H4 S32B
C
other Sb residues tested
156
D
156
other Sb residues tested

Figure 10. Fine mapping of Sb mAbs reveals that most cross-reactive mAbs are not sensitive to an E156K HA mutation.

(A, B) mAbs that were derived from mice immunized with PR8 and then boosted 28 days later with either PR8 (A) or S12a (B) were tested for binding to a panel of viruses. Twelve of these viruses had single point mutations at residues in and around the previously defined Sb site. Most S12a-reactive mAbs that were elicited by PR8-S12a sequential exposure were resistant to an E156K HA mutation (highlighted in red). More E156K-resistant mAbs were derived from PR8-S12a exposed mice compared to PR8-PR8 exposed mice (highlighted in red). (C, D) The locations of the point mutations tested in Figure S6A-B are highlighted in green on the HA structure shown from the side (C) and from the top (D). Residue 156 is highlighted in red.

Figure 11

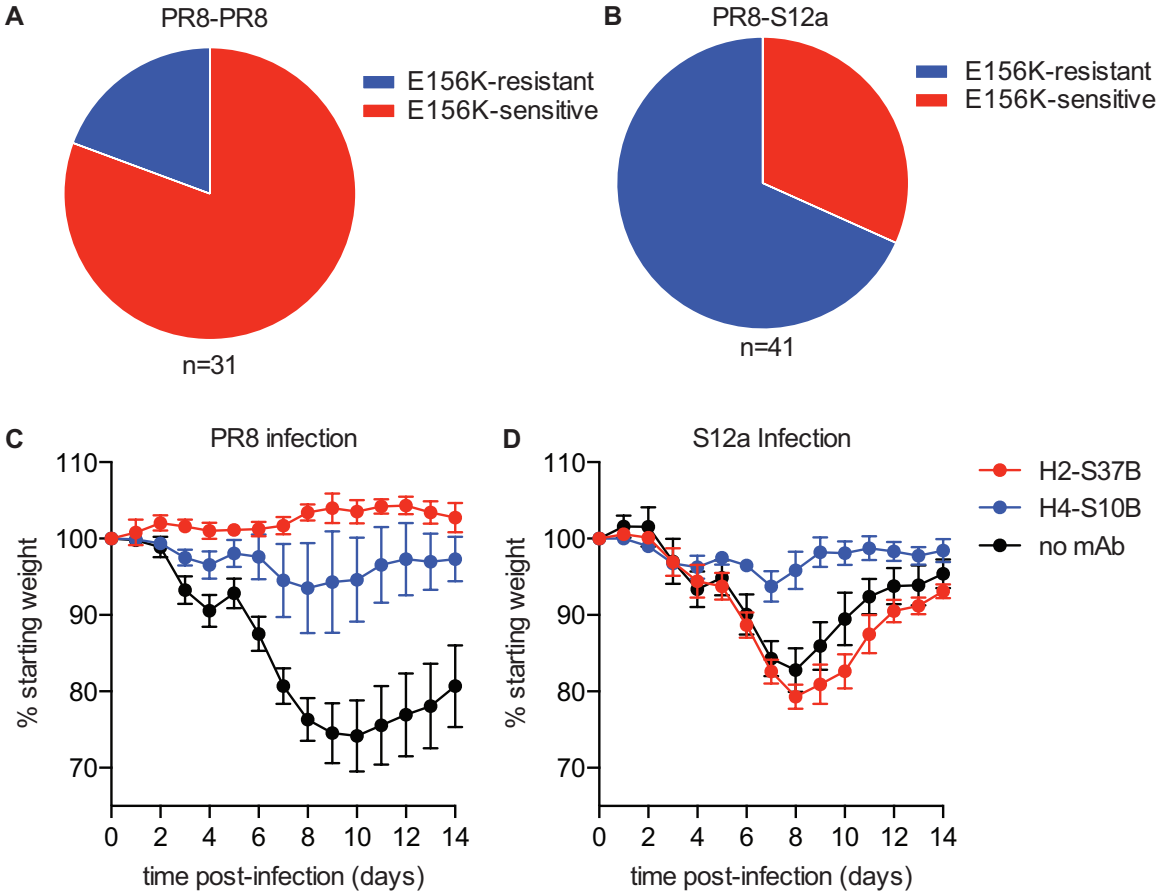


Figure 11. Most Sb mAbs elicited by PR8-S12a sequential exposure are not sensitive to the E156K mutations and protect against PR8 and S12a infection.

(A, B) Sb-specific mAbs from mice pre-exposed to PR8 virus and boosted with either PR8 (A) or S12a (B) virus were tested for binding by ELISA to PR8 viruses +/- the E156K HA mutation. mAbs that bound to the E156K mutant >25% relative to PR8 were considered 'E156K-resistant'. PR8-S12a exposure elicited a higher proportion of 'E156K-resistant' mAbs compared to PR8-PR8 exposure ($p < 0.0001$; Fisher's exact test). (C, D) 25ug of the H2-S37B or H4-S10B mAbs were transferred i.p. into mice 12 hours before infection with 30 TCID50 of PR8 (C) or S12a (D) virus. Weight loss was

measured for 14 days post-infection. PR8-infected mice that received either mAb lost significantly less weight than mice that received a PBS control (no mAb) ($p < 0.05$ at days 3-14 for H2 S37B, $p < 0.05$ at days 6-14 for H4 S10B; Student's t test). S12a-infected mice that received the H4-S10B mAb lost significantly less weight than mice that received a PBS control ($p < 0.05$ at days 7-9; Student's t test). Data are mean +/- SEM.

Figure 12

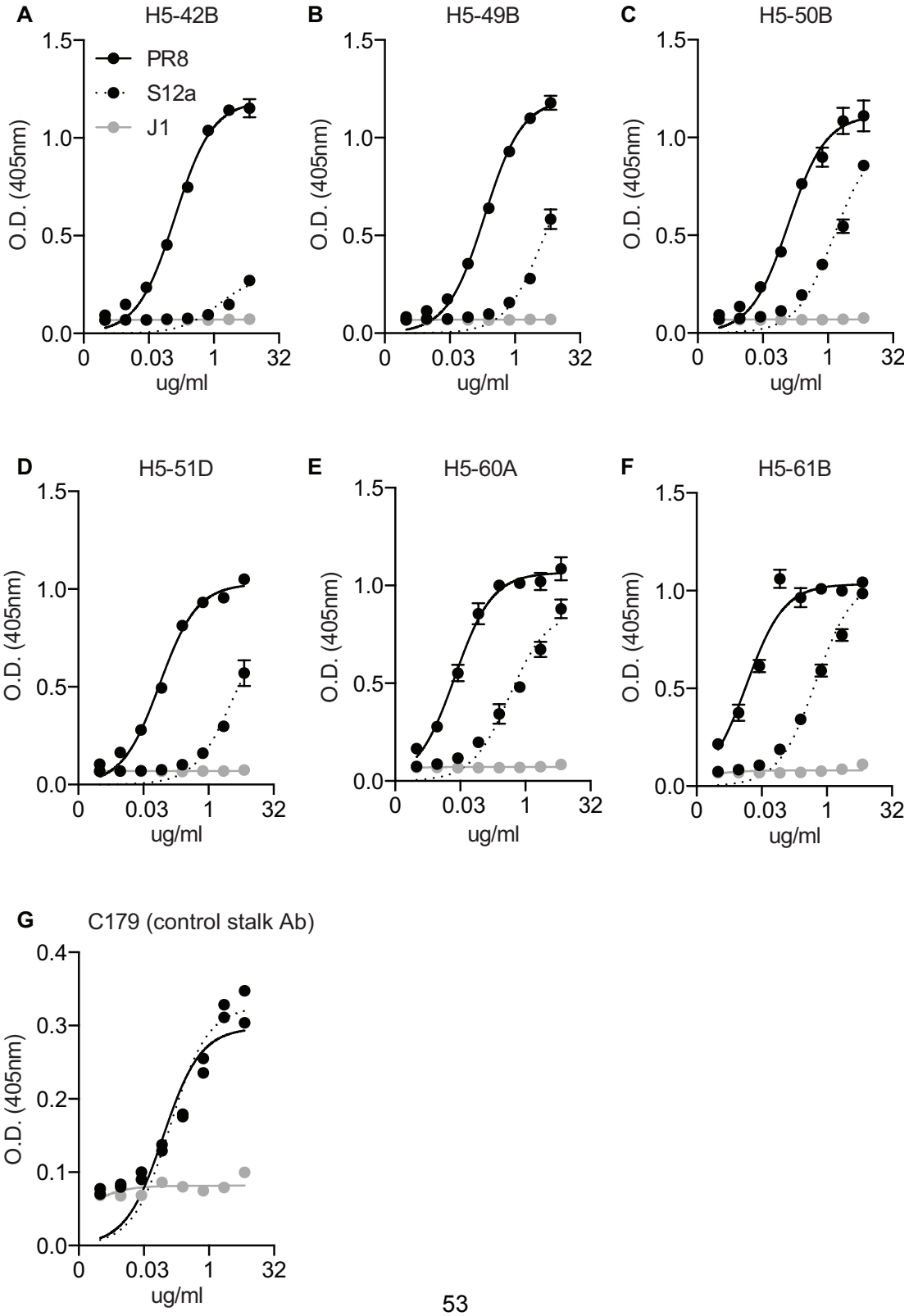

A
H5-42B
PR8
S12a
J1
O.D. (405nm)
ug/ml
B
H5-49B
C
H5-50B
D
H5-51D
E
H5-60A
F
H5-61B
G
C179 (control stalk Ab)
0
0.03
1
32
0.0
0.5
1.0
1.5
0.1
0.2
0.3
0.4

Figure 12. Somatic mutations affect binding to both PR8 and S12a.

(A-F) Six clonally related (see Table S1) IgG2a mAbs derived from a mouse primed with PR8 and boosted with S12a were tested for binding to PR8, S12a, and the antigenically unrelated J1 virus via ELISA (J1 is a virus that has an H3 HA). Data are mean +/- SEM. (G) As an ELISA coating control, the H1 stalk-reactive C179 mAb was tested for binding to PR8, S12a, and J1 virus. Data are mean +/- SEM.

Figure 13

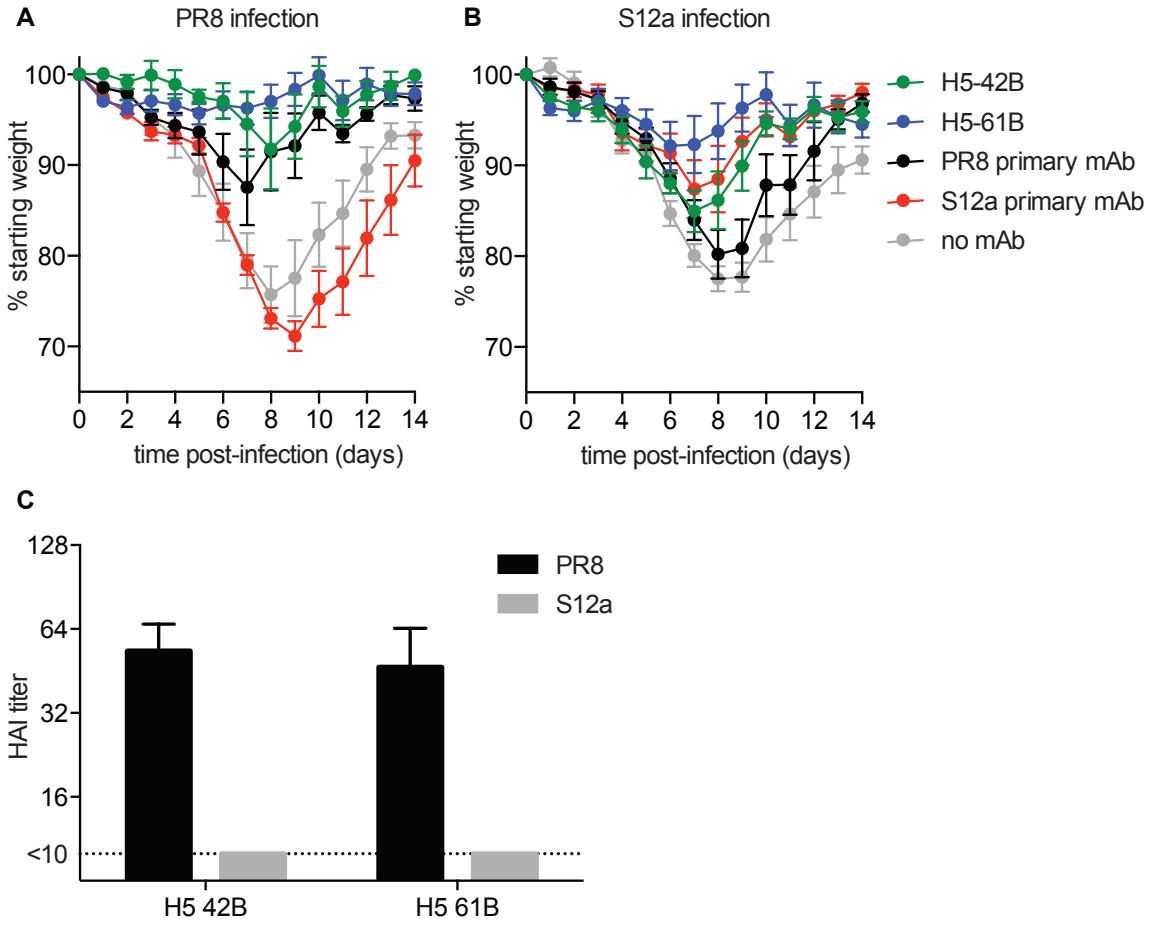


Figure 13. mAbs elicited by PR8-S12a immunization prevent disease caused by PR8 and S12a infections.

(A, B) 25ug of different mAbs were transferred i.p. into mice 12 hours before infection with 30 TCID50 PR8 (A) or S12a (B) (n=5 mice per group). PR8-infected mice that received H5-42B, H5-61B, or the PR8 primary mAb lost significantly less weight than mice that received a PBS control (no mAb) ($p < 0.05$ at days 5-14 for H5-42B, $p < 0.05$ at days 6-14 for H5-61B, $p < 0.05$ at days 8-10 for the PR8 primary mAb; Student's t

test). S12a-infected mice that received the H5-42B, H5-61B, or S12a primary mAb lost significantly less weight than mice that did not receive mAbs ($p < 0.05$ at days 8-12 for H5-42B, $p < 0.05$ at days 6-12 for H5-61B, $p < 0.05$ at days 6-14 for S12a primary mAb; Student's t test). (C) Sera was collected 12 hours after mAb transfer and tested by HAI assay against PR8 and S12a virus (n=3 mice per group). Data are mean +/- SEM.

Table 1

	CDR1				FR2	CDR2	FR3							CDR3			
AA residue	29	35	37	38	49	63	66	72	75	84	85	88	92	105	106	108	115
germline	ACT T	AGT S	TAT Y	TAC Y	AGG R	AGT S	TAC Y	AAG K	CGA R	AAG K	AAC N	TAC Y	AGC S	GCA A	AGA R	GAC N	ATG M
H5-61B	- - - 	- - - 	- T - F	- - - 	- - A 	- - - 	C - - H	C - - Q	- - - 	- G - R	- - - 	- - - 	- - - 	- T - V	- - G 	A - T 	- - -
H5-42B	- - - 	- - - 	- - - 	- - - 	- - - 	- - - 	- - - 	C - - Q	- - - 	- G - R	- - - 	- T - F	- - - 	T - - S	- - G 	A - T 	- - -
H5-49B	- - - 	- - - 	- - - 	- - - 	- - - 	- - - 	- - - 	C - - Q	- - - 	- G - R	- - - 	- - - 	- - - 	- T - V	- - G 	A - T 	- - -
H5-51D	T - - S	- - - 	- T - F	- - - 	- - - 	- - - 	- - T 	C - - Q	- - - 	- G - R	- - - 	- - - 	- - - 	- - - 	- - G 	A - T 	- - -
H5-50B	- - - 	- - C 	- T - F	- - T 	- - A 	- C - T	C - - H	C - - Q	- - C 	- G - R	GG - G	- - - 	- C - T	- - - 	- - G 	A - - 	- - -
H5-60A	- - - 	- - C 	- T - F	- - - 	- - A 	- - - 	C - - H	C - - Q	- - - 	- G - R	- - - 	- - - 	- - - 	- - - 	- - G 	A - - 	T - - L

Table 1: Identification of a clonally related family of mAbs.

Mutations that differ each mAb in the framework regions (FR) or complementarity-determining regions (CDR) from the heavy chain germline sequence (IGHV5-4*02, IGHD2-14*01, IGHJ4*01) are shown using IMGT (International immunogenetics information system) numbering (Giudicelli et al., 2011). FR1 was not determined because the sequencing primer bound to this region. We also sequenced the CDR3 region of the light chain (IGKV8-28*01, IGKJ5*01) which was identical between all 6 mAbs.

CHAPTER 3: POTENTIAL ANTIGENIC EXPLANATION FOR ATYPICAL H1N1 INFECTIONS AMONG MIDDLE-AGED ADULTS DURING THE 2013-2014 INFLUENZA SEASON

Summary

Influenza infections usually cause more severe disease in very young or elderly individuals. Intriguingly, H1N1 influenza infections caused an unusually high disease burden in middle-aged adults during the 2013-2014 influenza season. We hypothesize that this may be due to increased sensitivity of middle-aged adults to a new mutation that had recently been acquired by nearly all contemporary circulating H1N1 strains. To test this hypothesis, we tested sera from healthy human blood donors from different age groups against the newly mutated strain. Interestingly, we found that over 42% of individuals born between 1965 and 1979 exhibited reduced binding to the new mutated strain compared to the previously circulating strain. This was in stark contrast to younger individuals born between 1985 and 1997 who exhibited no reduction in titers against the newly mutated strain. We also found that mAbs isolated from middle-aged adults that bound to this epitope failed to protect mice from infection of the newly mutated strain. We show examples of middle-aged individuals who develop this particular specificity after immunization with the previously circulating strain and in ferrets sequentially exposed to strains relevant to middle-aged adults. This suggests that while sequential exposure shifts the Ab response to more conserved sites, this may ultimately lead to increased susceptibility when viral mutations in these sites occur.

Introduction

In the previous chapter, we have shown in a mouse model that sequential exposure to antigenically drifted strains of influenza virus elicits an increased proportion of cross-reactive Abs and that the specificity of mAbs shifts towards more conserved epitopes that are less sensitive to mutations that differ between the strains. Surprisingly, we also found that even OAS Abs that bind with a higher affinity to the priming strain can provide modest protection against influenza infections and may be precursors to more cross-reactive Abs though somatic hypermutation.

Unfortunately, influenza viruses are constantly mutating, and humans are potentially exposed to antigenically drifted viral strains on an annual basis. Shifts in epitope immunodominance have been shown to occur due to pre-exposure history (Li et al., 2013). As individuals from different age groups have different pre-exposure histories due to the persistent change in circulating viral populations, we hypothesize that this may explain why different age groups have different influenza virus-specific Ab repertoires. This suggests that while OAS Abs can have positive protective qualities due to limited cross-reactivity between two strains, the gradual shift of the Ab response to momentarily conserved epitopes may also lead to increased susceptibility should a virus arise that has mutations that abrogate Ab binding to a previously conserved region.

Results

An HA mutation acquired prior to the 2013-2014 influenza season is antigenically relevant in middle-aged adults

While the H1N1 component of the vaccine was not updated for the 2013-2014 season, mutations have been accumulating in circulating pH1N1 strains since the virus

arose in 2009 (Figure 14C). A K166Q mutation located on the distal head of the HA that was first sequenced from viral isolates in the 2012-2013 season became rapidly fixed in the circulating viral population (Figure 14A-B). This mutation has previously been considered antigenically neutral based on experiments using primary antisera from ferrets infected with the 2009 pH1N1 strain (Arriola, 2014). However, the antigenicity of this region may have been underrepresented by these experiments as primary antisera to the pH1N1 strain have been shown to be focused to other regions of the HA, particularly those epitopes containing residues 156, 157, and 158 (Chen et al., 2010; Li et al., 2013). The amino acids at residues 156, 157, and 158 have not been extensively mutated in pH1N1 strains circulating between 2009 and 2014 (Figure 15). In contrast to the ferrets used in surveillance experiments, humans are sequentially exposed to antigenically drifted strains throughout their lives. To test whether human sera binds to this newly mutated epitope, we tested sera collected from healthy human blood donors during the 2013-2014 influenza season by HAI against the wild type (WT) pH1N1 strain, and a pH1N1 strain engineered to contain the newly acquired K166Q mutation. Interestingly, we found that 27% of sera from individuals born between 1940 and 1984 had at least a twofold reduction in titers to the new mutant virus when compared to the WT strain. Remarkably, over 42% of individuals born from 1965 to 1979 (n = 54 individuals) also demonstrated this reduction. This is in contrast to younger individuals born from 1985 to 1997 (n = 49 individuals) who did not have a measurable decrease in serum titers to the newly mutated strain (Figure 16A, Table 2). This difference in K166 specificity between these two age groups was statistically significant ($p < 0.0001$; Fisher's exact test). Similar results were obtained when we tested sera from healthy human adults collected during the 2013-2014 influenza season in Mexico (Figure 17, Table 3).

The difference in K166 epitope immunodominance is due to unique pre-exposure history

We hypothesize that the difference between these two age groups is due to a difference in pre-exposure history. We propose that the Abs elicited in middle-aged adults that are specific for this newly mutated epitope were elicited by previously circulating sH1N1 strains circulating in humans before 1985 and then recalled by the 2009 pH1N1 strain. sH1N1 strains that circulated in the late 1970s and early 1980s share extensive homology with the pH1N1 strain (Figure 16B) and H1N1 strains did not circulate in humans from 1957 to 1976, so adults born after 1957 would have been potentially exposed to H1N1 strains as early as 1977. In contrast, sH1N1 strains acquired a mutation that introduced a glycosylation site at HA residue 129. This glycosylation site blocks binding of K166-specific mAbs (Linderman et al., 2014). We hypothesize that K166 epitope specific Abs were not elicited by seasonal strains circulating after 1985 when the glycosylation site that shields this epitope was introduced and so were not recalled by infection or immunization with the pH1N1 strain that lacks this glycosylation. To test our hypothesis that sequential exposure to strains circulating before 1985 and the 2009 pH1N1 strain affected the prevalence of K166-specific Abs, we tested sera from humans born before 1984 that was collected before and after vaccination with the 2009 pH1N1 strain. As these sera were collected in 2009, most individuals did not have measurable titers to the 2009 pH1N1 strain before vaccination. Sera from 5 out of 17 individuals demonstrated a measurable increase in K166 epitope specific titers after vaccination with the 2009 pH1N1 (Figure 18A, Table 4). We also measured binding of 42 head specific mAbs collected from 12 adult donors (born 1949-1985) that were vaccinated with the pH1N1 strain in 2009. Remarkably, 23% of these mAbs had reduced binding to a pH1N1 K166Q mutant virus compared to the WT 2009

pH1N1 strain (Figure 18B). We hypothesize that these Abs were previously elicited by seasonal strains and recalled by the 2009 pH1N1 vaccination. To test this in a more controlled animal model, we sequentially infected ferrets with sH1N1 strains that circulated in 1977, 1983, or 1986, followed by infection with the 2009 pH1N1 strain. As controls, we infected previously naïve ferrets with the 2009 pH1N1 strain and also sequentially infected ferrets with the 2009 pH1N1 strain twice. Three out of eight ferrets that were infected with the pH1N1 strain after pre-exposure to the A/Chile/01/1983 virus had detectable K166 specific titers (Figure 19, Table 5). This was in contrast to ferrets pre-exposed to the A/Singapore/06/1986 strain which has a glycosylation site that is predicted to shield the region around residue 166, as well as ferrets pre-exposed to the A/USSR/90/1977 strain. The A/USSR/90/1977 - A/California/07/2009 strain likely did not recall K166 HA specific mAbs because of variation at residue 125, which is in close proximity to residue 166 (Figure 20). Neither ferrets that received one pH1N1 infection or two sequential pH1N1 infections elicited detectable K166 HA specific titers.

K166 HA-specific Abs are less effective against K166Q HA mutant virus infection

In the previous chapter, we showed that even Abs with low affinity for a mutated strain could protect against infection. We passively transferred a K166 HA specific mAb (SFV009—3F05) or a control mAb that binds equally well to the K166Q HA mutant and WT strains (SFV015-1F02) to BALB/c mice 12 hours prior to infection with the WT pH1N1 strain or a K166Q HA mutant strain (Figure 18C). While both mAbs protected against the WT pH1N1 strain, the K166 HA specific mAb failed to protect mice against infection with the K166Q HA mutant strain. In addition, we tested human sera by *in vitro* neutralization assay. Sera that had K166 HA specificity based on HAI assays failed to efficiently neutralize K166Q HA mutant viruses (Table 6). This suggests that K166 HA

specific Abs can be less effective at preventing disease. As we have shown that these types of mAbs are more common in middle-aged adults, this offers a potential antigenic explanation for the increased disease burden in middle-aged adults that occurred during the 2013-2014 influenza season.

Discussion

Our studies suggest that an HA mutation that was first recognized during the 2012-2013 influenza season is antigenically relevant and potentially physiologically relevant in a subset of the population. The antigenic change caused by the K166Q HA mutation may have been previously overlooked as primary ferret sera to the pH1N1 strain do not represent the influenza-specific Ab repertoire in humans who are exposed to influenza viruses repeatedly throughout life. To complicate matters, since influenza viruses are constantly mutating, individuals from different age groups have unique pre-exposure histories. We hypothesize that this affects the influenza-specific Ab repertoire and may explain the unusually high level of disease in middle-aged adults during the 2013-2014 influenza season. We have shown here that the K166Q HA mutation abrogates binding of Abs prevalent in middle-aged adults. Previous studies have shown that sequentially infecting ferrets with antigenically drifted strains of influenza shifts the influenza-specific Ab response to focus on different epitopes (Li et al., 2013). We have recapitulated this and found that some ferrets pre-exposed to a previously circulating seasonal strain elicit a strong Ab response against this newly mutated region of the HA. In addition, we have found that vaccination with the pH1N1 strain can increase the titers of Abs of this specificity. These Abs have reduced neutralization capabilities against the newly mutated strain. This suggests that surveillance of influenza viruses should take into account the effect of pre-exposure history on the influenza-specific Ab repertoire

when considering whether a new mutation that arises in the circulating viral population constitutes an antigenic change. In addition, these studies suggest that pre-exposure history not only affects the Ab repertoire, but may also affect susceptibility to influenza infection.

Materials and Methods

Human Donors

Studies involving human adults were approved by the Institutional Review Boards of Emory University, Vaccine and Gene Therapy Institute of Florida, the National Institute of Respiratory Diseases of Mexico, and the Wistar Institute. Informed consent was obtained. For all experiments, HAI and *in vitro* neutralization assays were completed at the Wistar Institute using preexisting and de-identified sera. We analyzed several serum panels in this study. We analyzed sera from healthy donors collected at the New York Blood Center in February of 2014. We analyzed sera from healthy donors collected at the Center for Research in Infectious Diseases at the National Institute of Respiratory Diseases in Mexico. We analyzed sera and mAbs derived from healthy donors vaccinated with a monovalent pH1N1 vaccine in 2009 as previously described (Li et al., 2012).

Viruses

Viruses possessing WT pH1N1 HA or K166Q pH1N1 HA were generated via reverse-genetics using HA and NA genes from A/California/07/2009 and internal genes from A/Puerto Rico/08/1934. All of these viruses were engineered to possess the antigenically neutral D225G HA mutation, which facilitates viral growth in fertilized

chicken eggs (Chen et al., 2010). Viruses were grown in fertilized chicken eggs and the HA genes of each virus stock were sequenced to verify that additional mutations did not arise during propagation. sH1N1 strains (A/USSR/90/1977, A/Chile/01/1983, A/Singapore/06/ 1986, A/Texas/36/1991, A/New Caledonia/20/1999, and A/Solomon Islands/03/2006) were also grown in fertilized chicken eggs. We isolated a pH1N1 virus from respiratory secretions obtained from a patient from the Children's Hospital of Philadelphia in 2013 (A/CHOP/1/13). For this process, de-identified clinical material from the Children's Hospital of Philadelphia Clinical Virology Laboratory was added to MDCK cells (originally obtained from the National Institutes of Health) in serum-free media with TPCK-treated trypsin (Worthington), HEPES (Cellgro), and gentamicin (Gibco). Virus was isolated from the MDCK-infected cells 3 days later. We extracted viral RNA and sequenced the HA gene of A/CHOP/1/13.

Animal Experiments

Murine experiments were performed at the Wistar Institute according to protocols approved by the Wistar Institute Institutional Animal Care and Use Committee. BALB/c mice (Charles River Laboratories) were injected with 25 μg of mAb i.p. and then infected intranasaly with 20,000 TCID50 of WT or K166Q-HA pH1N1 virus 12 hours later. As controls, some mice received an i.p injection of PBS (Cellgro) before infection. Weight loss and survival was recorded for 11 days. Severely sick mice were euthanized. Ferret experiments were performed at the Vaccine and Gene Therapy Institute of Florida in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories handbook (Council, 2011). Fitch ferrets (Marshall Farms) were infected with 1×10^6 PFU of sH1N1 virus and bled 14 and 84

days later. These ferrets were then infected with the A/California/ 07/2009 pH1N1 strain and bled 14 days later. Some ferrets were sequentially infected with A/California/07/2009 (84 days between infections) and other ferrets were infected with only A/California/07/2009 and bled 14 days later.

HAI Assays

Sera samples were pretreated with receptor-destroying enzyme (Key Scientific Products or Sigma-Aldrich) and HAI titrations were performed in 96-well round bottom plates (BD). Sera were serially diluted twofold and added to four agglutinating doses of virus in a total volume of 100 µL. Turkey erythrocytes (Lampire) were added [12.5 µL of a 2% (vol/vol) solution]. The erythrocytes were gently mixed with sera and virus and agglutination was read out after incubating for 60 minutes at room temperature. HAI titers were expressed as the inverse of the highest dilution that inhibited four agglutinating doses of turkey erythrocytes. Each HAI assay was performed independently on three different dates. Sera that had at least twofold reduced HAI titers using K166Q HA mutant viruses in three independent HAI assays were labeled as "K166 HA-specific."

ELISA

Viruses for ELISAs were concentrated by centrifugation at 20,000 RPM for 1 hour using a Thermo Scientific Sorvall WX Ultra 80 Centrifuge with a Beckman SW28 rotor. Concentrated viruses were then inactivated by BPL (Sigma-aldrich) treatment. Viruses were incubated with 0.1% BPL and 0.1M HEPES overnight at 4C followed by a 90 minute incubation at 37 °C. The 96-well Immulon 4HBX flat-bottom microtiter plates (Fisher Scientific) were coated with 20 HAU per well BPL-treated virus overnight at 4 °C.

Each human mAb was serially diluted in PBS and added to the ELISA plates and allowed to incubate for 2 hours at room temperature. As a control, we added the 70-1C04 stalk-specific mAb to verify equal coating of WT and K166Q HA virus. Next, peroxidase conjugated goat anti-human IgG (Jackson Immunoresearch) was incubated for 1 hour at room temperature. Finally, Sureblue TMB Peroxidase Substrate (KPL) was added to each well and the re-action was stopped with addition of 250 mM HCl solution. Plates were extensively washed with water between each ELISA step. Affinities were determined by nonlinear regression analysis of curves of 6 mAb dilutions (18 μg/mL to 74 ng/mL) using Graphpad Prism. mAbs were designated as K166 specific if they had a K_d at least four times greater for the K166Q mutant than for the WT virus.

In Vitro Neutralization Assay

Sera were serially diluted and then added to 100 TCID50 units of virus and incubated at room temperature for 30 minutes. The virus-serum mixtures were then incubated with MDCK cells for 1 hour at 37 °C. Cells were washed and then serum-free media with TPCK-treated trypsin was added. Endpoints were determined visually 3 days later. Data are expressed as the inverse of the highest dilution that caused neutralization. All samples were repeated in quadruplicate and geometric mean titer is reported.

Structural Modeling of HA Glycosylation Sites

Glycans were modeled onto positions 129 and 131 in the A/Solomon Islands/03/2006 HA crystal structure (PDB ID code 3SM5) using the GLYCAM Web Glycoprotein Builder (www.glycam.org). The particular glycan used for modeling was an N-linked glycan with a trimannosyl core (DManpa1-6[DGlcpNAcb1-2DManpa1-

3]DManpb1-4DGlcpNAcb1- 4DGlcpNAcb1-OH in Glycam notation), and default rotamer settings were used for modeling. To model the 131 glycosylation site, a T131N mutation was introduced using the PyMol structure viewer before the structure was uploaded to the GLYCAM-Web server.

Computational and Phylogenetic Analyses of HA Sequences

The occurrence of different amino acid identities at HA residues 166, 156, 157, and 158 (H3 numbering) was analyzed by downloading all full-length human pandemic H1N1 sequences present in the Influenza Virus Resource as of February 23, 2014 (Bao et al., 2008). After purging sequences that were less than full length, contained ambiguous nucleotide identities, lacked full (year, month, day) isolation dates, or were otherwise anomalous, the sequences were aligned. Each calendar year was broken into four equal partitions beginning with January 1, and the frequencies of different amino acids at each residue of interest for each partition was calculated and plotted. Only amino acids that reached a frequency of at least 1% in at least one of the year partitions are labeled in the legend to the plot. For construction of phylogenetic trees, the sequence set was randomly subsampled to 10 sequences per quarter-year partition. BEAST was then used to sample from the posterior distribution of phylogenetic trees with reconstructed sequences at the nodes, after date stamping the sequences, using a JTT with a single rate category with an exponential prior, a strict molecular clock, and relatively uninformative coalescent-based prior over the tree (Drummond et al., 2012; Jones et al., 1992). Fig. 1C shows a maximum clade credibility summary of the posterior distribution with branches colored according to the reconstructed amino acid identity at site 166 with the highest posterior probability at their descendent nodes. The tree was visually rendered using FigTree. The input data and computer code used for

this analysis can be found on GitHub at github.com/jbloom/pdmH1N1_HA_K166_mutations.

Statistical Analyses

For all serum experiments, we excluded samples that did not have positive pH1N1 HAI titers. All samples that were pH1N1 HA-WT HAI negative were also pH1N1 HA-K166Q HAI-negative. Samples were allocated to specific groups based on age of donor. The year of birth of each sample was available during the experiment, but this information was not assessed until each experiment was completed. Variance of raw HAI titers was similar between different age groups. Fisher's exact tests were completed using SAS v9.3 software.

Figures and Tables

Figure 14

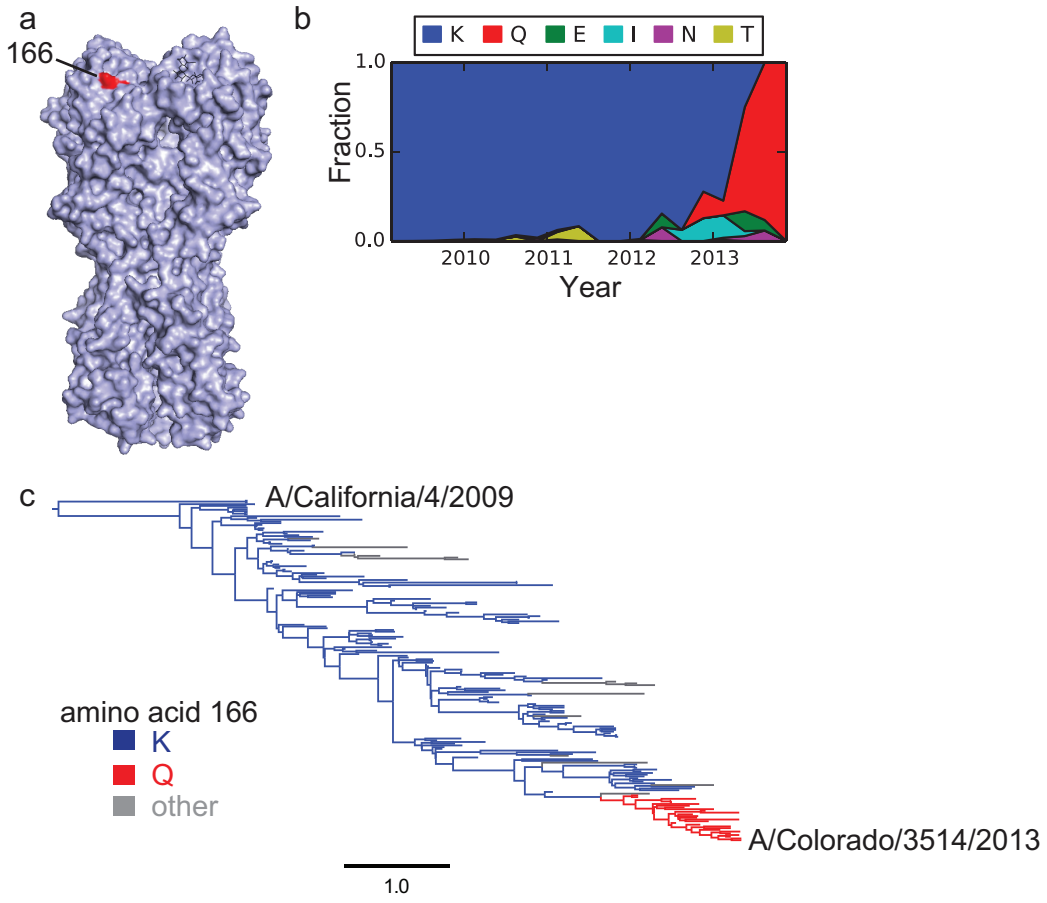


Figure 14. pH1N1 viruses rapidly acquired HA mutation K166Q during the 2013–2014 influenza season.

(A) Residue K166 (red) is shown on the A/California/04/2009 HA trimer [PDB ID code 3UBN (Xu et al., 2010)]. (B) Plotted is the frequency of different amino acid identities at HA residue 166 in pH1N1 HA sequences as a function of time. Nearly all pH1N1 possessed K166 from 2009 to mid-2012, but most isolates possessed Q166 by the 2013–2014 season. (C) A phylogenetic tree of pH1N1 viruses with branches colored

according to amino acid identity at site 166 illustrates the rapid fixation of K166Q in recent pH1N1 isolates.

Figure 15

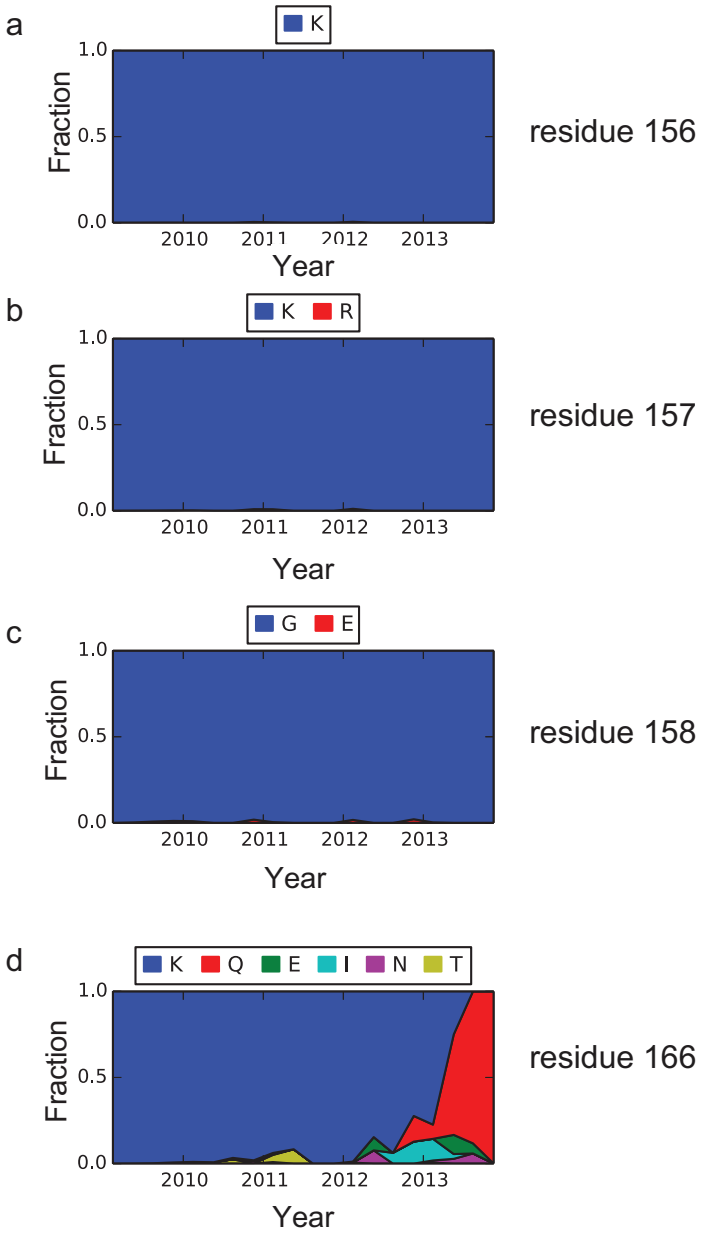


Figure 15. Sequence variation of pH1N1 HA.

The residues in the dominant antigenic site recognized by primary ferret anti-sera (residues 156, 157, and 158 of the Sa antigenic site) are highly conserved in pH1N1 (A–C). No variation greater than 1% occurred at residue 156 and very little variation

occurred at residues 157 and 158. For comparison, residue 166 (D; also shown in Fig. 1B) has undergone a complete change in the last year.

Figure 16

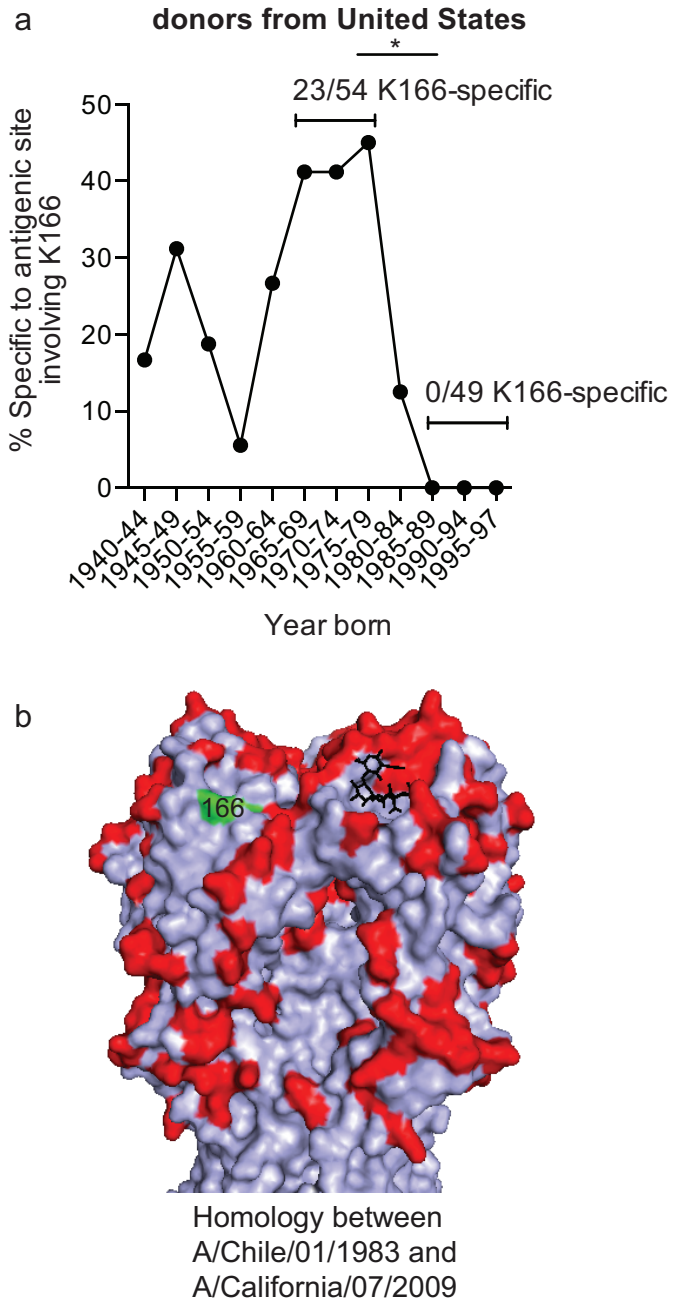


Figure 16. Adult humans possess Abs that bind to a region of HA that was recently mutated in pH1N1.

(A) Sera were isolated from healthy donors (n = 195) from the state of New York during

the 2013–2014 influenza season. HAI assays were performed using viruses with either WT A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. For each sera sample, we completed three independent HAI assays. Raw HAI data are reported in Table 2. Percentages of samples that had at least a twofold reduction in HAI titer using the mutant virus in three independent experiments are shown. K166 specificity of sera from individuals born between 1965 and 1979 is statistically significant compared with K166 specificity of sera from individuals born after 1985 (*$p < 0.0001$; Fisher's exact test). (B) Homology between the A/Chile/01/1983 sH1 and the A/California/04/2009 pH1 are shown using the crystal structure of the A/California/04/2009 HA [PDB ID code 3UBN (Xu et al., 2010)]. Residue K166 is colored green. Amino acids that differ between A/Chile/01/ 1983 and A/California/04/2009 are shown in red. The glycan receptor is shown in black.

Figure 17

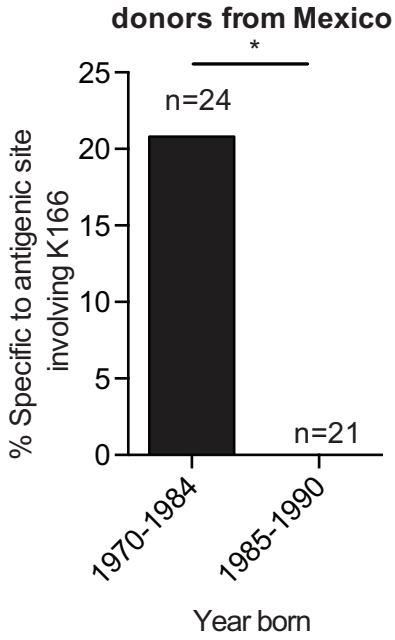


Figure 17. Mexican donors born before 1985 possess Abs that bind to the region of HA that was recently mutated in pH1N1.

Sera were isolated from healthy donors (n = 45) at the Center for Research in Infectious Diseases at the National Institute of Respiratory Diseases in Mexico during the 2013–2014 influenza season. HAI assays were performed using viruses with either WT A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. For each sera sample, we completed three independent HAI assays. Raw HAI data are reported in Table 3. Percentages of samples that had at least a twofold reduction in HAI titer using the mutant virus in three independent experiments are shown. (*$p < 0.05$; Fisher's exact test).

Figure 18

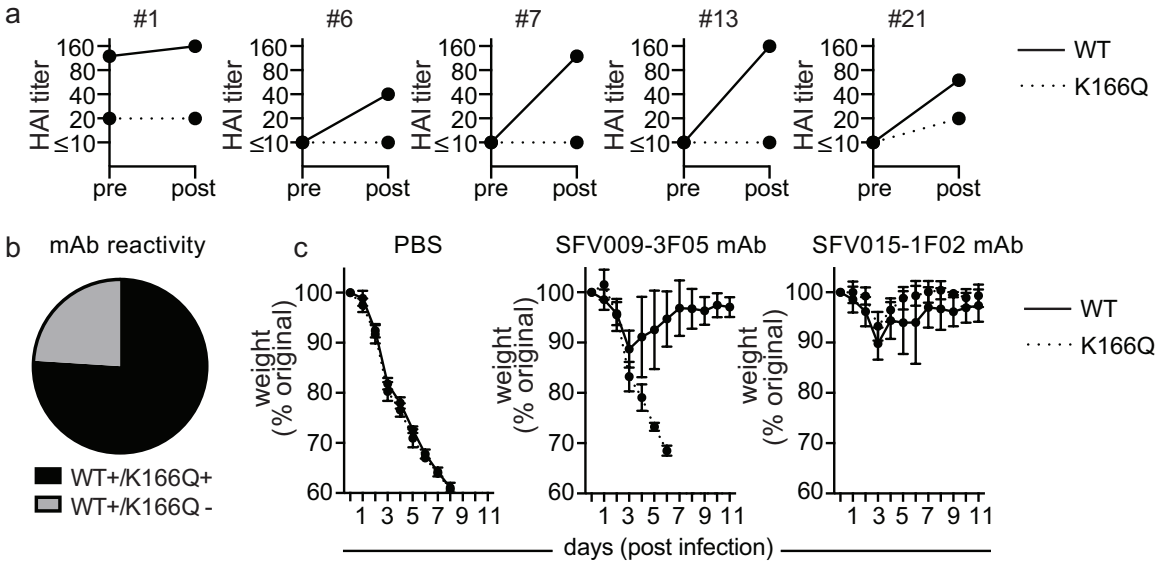


Figure 18. Vaccination of middle-aged adults with the current pH1N1 vaccine strain elicits Abs that bind to a region of HA that is now mutated in most pH1N1 isolates.

(A) Healthy adult volunteers were vaccinated with a monovalent pH1N1 vaccine in 2009. Sera were isolated pre-vaccination and 30 days post-vaccination and HAI assays were performed using viruses with either WT A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. Shown are HAI titers for donors that possessed K166 HA-specific Abs following vaccination. Data are representative of three independent experiments. Raw HAI titers for all donors are shown in Table 4. (B) ELISAs were completed using mAbs isolated from healthy adult volunteers that were vaccinated with a monovalent pH1N1 vaccine in 2009. ELISAs were coated either with A/California/07/2009 (WT) or A/California/07/2009 with a K166Q HA mutation. Shown are percentage of mAbs that bound to both viruses and percentage of mAbs that bound

to the WT virus but not the mutant virus (n = 42 mAbs). Data are representative of two independent experiments. (C) A K166 HA specific mAb (SFV009-3F05) or a mAb that recognizes both WT and K166Q HA pH1N1 (SFV015-1F02) were injected into BALB/c mice (n = 4 per group). Twelve hours later, mice were then infected with 20,000 TCID50 of WT or K166Q HA virus and weight loss and survival were recorded for 11 days. Data are representative of two independent experiments.

Figure 19

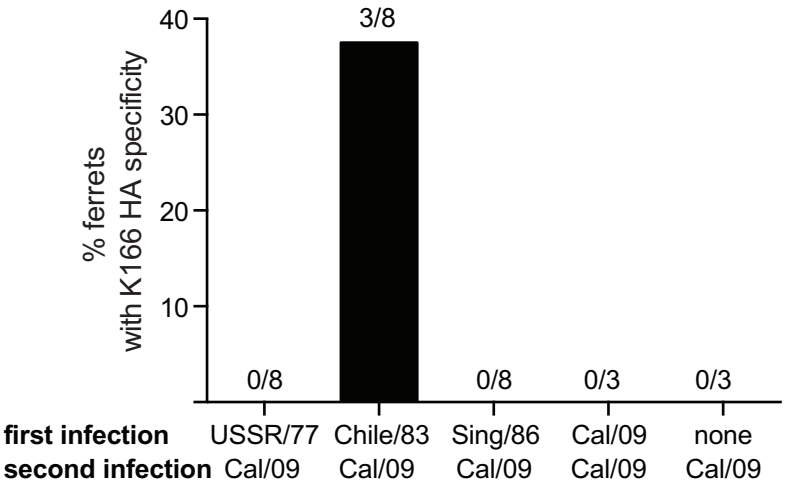


Figure 19. Ferrets sequentially infected with A/Chile/01/1983 and A/California/ 07/2009 develop K166 HA specific Abs.

Ferrets were infected with a sH1N1 virus and then reinfected 84 days later with the A/California/07/2009 pH1N1 virus. Sera were collected 14 days after the second infection and HAI assays were completed using WT and K166Q-HA pH1N1 viruses. Shown are percentages of samples that had at least a twofold reduction in HAI titer using the K166Q HA mutant virus in three independent experiments. Raw HAI titers are shown in Table 5. The difference in K166 HA specificity is statistically significant comparing the A/Chile/01/1983-A/California/07/2009 group with the rest of the groups (3 of 8 vs. 0 of 22; $p < 0.05$; Fisher's exact test).

Figure 20

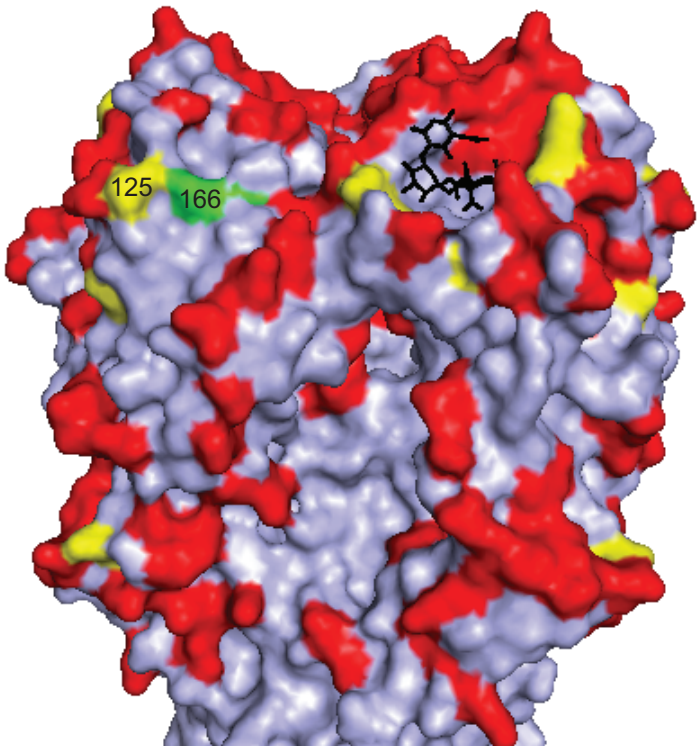


Figure 20. Homology between A/USSR/90/1977, A/Chile/01/1983, and A/California/07/2009.

HA residues that differ between A/Chile/01/1983 and the A/California/ 07/2009 are shown in red. A few additional HA residues differ between the HAs of A/USSR/90/1977 and A/California/7/2009, and these are colored yellow. Of note, A/Chile/01/1983 and A/California/07/2009 both possess S125 whereas A/USSR/90/1977 possess R125. Residues 125 and 166 are next to each other in the structure. PDB ID file 3UBN (A/California/04/2009 HA) was used to make this figure.

Table 2

		experiment #1		experiment #2		experiment #3	
		HAI titers		HAI titers		HAI titers	
sample ID	YOB	WT	K166Q	WT	K166Q	WT	K166Q
40-60	1940	160	80	160	80	160	60
40-49	1943	80	<20	60	<20	80	<20
40-05	1948	160	80	160	80	160	80
40-02	1948	120	60	60	30	80	30
40-44	1948	320	80	320	80	320	80
40-14	1949	240	80	240	80	240	80
40-16	1949	240	<20	160	<20	160	<20
50-55	1952	160	80	160	60	160	80
50-11	1952	120	40	80	30	80	30
50-43	1953	80	40	80	40	80	40
50-06	1959	120	60	80	40	80	30
60-04	1963	80	40	80	40	80	40
60-10	1963	160	80	240	80	160	60
60-13	1964	240	120	320	120	240	120
60-25	1964	60	20	80	30	60	20
60-14	1967	160	80	120	60	120	60
60-23	1967	320	120	320	160	320	160
60-44	1968	160	80	160	80	160	80
60-28	1969	320	160	320	160	320	160
60-41	1969	120	60	160	80	160	80
60-63	1969	120	60	120	60	120	60
60-35	1969	80	40	80	30	80	30
70-56	1971	80	40	80	30	80	40
70-52	1971	160	<20	120	<20	80	<20
70-60	1972	160	80	160	80	160	80
70-74	1972	160	80	240	80	240	80
70-36	1972	240	60	240	30	320	30
70-84	1973	120	40	160	30	160	40
70-86	1973	160	40	160	30	160	40
70-13	1975	640	320	640	320	640	320
70-54	1975	160	80	80	30	120	60
70-25	1975	160	80	80	30	120	40
70-10	1975	160	30	80	30	80	30
70-23	1975	160	<20	120	<20	160	<20
70-82	1976	160	80	160	60	160	80
70-50	1976	240	80	160	60	120	60
70-80	1978	320	160	320	160	320	160
70-19	1978	80	<20	80	<20	80	<20
80-16	1980	80	30	60	20	80	40
80-48	1983	640	120	640	80	640	80

Table 2: HAI titers using sera from healthy donors from the United States.

Sera were isolated from 195 healthy donors from the state of New York during the 2013-2014 influenza season. HAI assays were performed using viruses with either wild-type A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. For each serum sample, we completed 3 independent HAI assays. Shown are samples that had at least a twofold reduction in HAI titer using the mutant virus in 3 independent experiments.

Table 3

		experiment #1		experiment #2		experiment #3	
		HAI titers		HAI titers		HAI titers	
sample ID	YOB	WT	K166Q	WT	K166Q	WT	K166Q
N-33	1973	160	60	120	40	80	30
N-42	1975	40	<10	30	<10	30	<10
N-93	1977	120	30	60	30	60	20
N-72	1979	30	<10	30	<10	20	<10
N-70	1980	160	80	120	60	120	40

Table 3: HAI titers using sera from healthy donors from Mexico.

Sera were isolated from 45 healthy donors from Mexico during the 2013-2014 influenza season. HAI assays were performed using viruses with either wild-type A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. For each serum sample, we completed 3 independent HAI assays. Shown are samples that had at least a 2-fold reduction in HAI titer using the mutant virus in 3 independent experiments.

Table 4

K166-specific donors

		visit 1 (pre-vaccine)							visit 2 (post-vaccine)	
I.D.	YOB	USSR/77	Chile/83	Texas/91	NC/99	SI/06	CAL/09	CAL/09-K166Q	CAL/09-WT	CAL/09-K166Q
1	1979	240	160	320	60	80	120	20	160	20
6	1966	160	120	240	120	240	<10	<10	40	10
7	1966	40	60	30	10	60	<10	<10	120	<10
13	1982	80	40	80	40	60	10	<10	160	10
21	1958	60	30	80	40	80	<10	<10	60	20

other donors

		visit 1 (pre-vaccine)							visit 2 (post-vaccine)	
I.D.	YOB	USSR/77	Chile/83	Texas/91	NC/99	SI/06	CAL/09	CAL/09-K166Q	CAL/09-WT	CAL/09-K166Q
2	1976	80	80	120	<10	<10	60	60	80	60
5	1949	10	<10	<10	10	40	10	<10	40	40
8	1961	40	<10	30	80	120	<10	<10	80	80
9	1977	80	80	80	<10	<10	<10	<10	120	80
10	1983	80	<10	320	240	320	<10	<10	240	320
15	1961	<10	<10	<10	<10	<10	<10	<10	80	80
17	1956	40	<10	30	80	120	10	<10	20	20
19	1961	80	40	160	80	80	<10	<10	20	20
20	1945	40	<10	<10	<10	<10	<10	<10	240	320
22	1956	30	30	60	<10	<10	<10	<10	240	240
23	1975	40	<10	240	80	120	<10	<10	160	160
24	1968	<10	<10	30	<10	<10	<10	<10	60	60

Table 4: Vaccination elicits K166 HA specific responses.

HAI assays were completed using sera isolated from healthy donors before and after vaccination with the monovalent pH1N1 virus in 2009. Post-vaccination sera were collected 30 days after vaccination. HAI assays using post-vaccine sera were completed 3 independent times and assays using pre-vaccine sera were completed 2 independent times.

Table 5

First Infection	second infection	animal	day 14		day 84		day 98	
			WT	K166Q	WT	K166Q	WT	K166Q
A/USSR/90/77	A/CA/07/09	7982	<10	<10	<10	<10	800	800
		7983	<10	<10	<10	<10	800	800
		7984	<10	<10	<10	<10	300	300
		7985	<10	<10	<10	<10	800	600
		7991	<10	<10	<10	<10	2400	2400
		7993	<10	<10	<10	<10	800	1200
		7994	<10	<10	<10	<10	600	600
		7995	<10	<10	<10	<10	400	600
A/Chile/01/83	A/CA/07/09	7987	<10	<10	<10	<10	1200	800
		7988	<10	<10	<10	<10	800	400
		7989	<10	<10	<10	<10	800	400
		7990	<10	<10	<10	<10	1600	1600
		7996	<10	<10	<10	<10	1200	400
		7997	<10	<10	<10	<10	240	160
		8000	<10	<10	<10	<10	600	800
		8001	<10	<10	<10	<10	800	800
A/SI/06/86	A/CA/07/09	1487	<10	<10	80	120	6400	6400
		1488	<10	<10	<10	<10	3200	3200
		1481	<10	<10	<10	<10	2400	3200
		1504	<10	<10	60	80	4800	6400
		8002	<10	<10	<10	<10	1600	2400
		8009	<10	<10	<10	<10	1600	2400
		8010	<10	<10	<10	<10	4800	6400
		8012	<10	<10	<10	<10	1600	2400
A/CA/07/09	A/CA/07/09	8013	400	400	200	200	160	160
		8014	800	800	400	400	2400	2400
		8015	800	800	300	200	2400	1600
None	A/CA/07/09	1486	1600	2400	ND	ND	ND	ND
		1497	1600	1600	ND	ND	ND	ND
		1492	1200	1600	ND	ND	ND	ND

Table 5: Sequential infection in ferrets can elicit K166 HA specific Abs.

Ferrets were infected with a sH1N1 virus and bled 14 and 84 days later. Animals were then infected with a pH1N1 strain and bled 14 days later (98 days post first infection). Sera were isolated and HAI assays were completed using WT and K166Q HA pH1N1 viruses. Data are representative of three independent HAI assays. The three ferrets with K166 HA specific Ab responses had >twofold changes in HAI titer using the K166Q virus in three independent experiments. Red highlights twofold or greater reductions in HAI titer using K166Q HA pH1N1 virus compared to WT HA pH1N1 virus. ND, not determined.

Table 6

		HAI titers		*in vitro* neutralization titers	
sample #	YOB	A/CA/07/09-WT HA	A/CHOP/1/2013	A/CA/07/09-WT HA	A/CA/07/09-K166Q HA
40-49	1943	20	<10	24	12
40-44	1948	160	30	453	95
40-16	1949	80	<10	160	20
70-52	1971	40	<10	190	28
70-36	1972	120	10	381	95
70-84	1973	40	<10	160	57
70-86	1973	60	20	190	67
70-23	1975	80	<10	226	20
70-19	1978	30	<10	95	10
80-48	1983	320	30	1076	80
80-10	1986	160	160	761	1810
80-26	1986	80	80	113	160
80-74	1986	40	40	135	113
80-30	1987	20	10	67	113
80-34	1987	160	160	381	538
80-56	1988	20	<10	17	34
90-01	1990	40	10	48	80
90-24	1991	60	20	34	95
90-12	1992	160	60	190	226
90-13	1992	80	80	190	320

Table 6: Characterization of K166 HA-specific sera.

We further characterized several serum samples that had immunodominant K166 HA specific Abs (light grey = K166 HA specific sera from individuals born prior to 1985; darker grey = sera from individuals born after 1985). HAI assays were completed using

viruses with the A/California/07/2009 HA-WT and a primary pH1N1 virus isolated from the Children's Hospital of Philadelphia (CHOP) in 2013. Additional *in vitro* neutralization assays were completed using the reverse-genetics derived viruses. Data are representative of 2 independent experiments. For each neutralization assay, each sample was titered 4 times, and geometric mean of these quadruplicate samples is reported.

CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS

It has long been held that exposure to an antigen leads to an anamnestic response that can provide protection. Thucydides recorded that in the second year of the Peloponnesian war (430 BCE) Athens suffered from a terrible plague, and that the sick found the most help from those who had recovered from the disease as these "had now no fear for themselves; for the same man was never attacked twice - never at least fatally. And such persons not only received the congratulations of others, but themselves also, in the elation of the moment, half entertained the vain hope that they were for the future safe from any disease whatsoever" (Thucydides, 431 BCE). This observation of protection has lead to the development of vaccines, most of which are thought to function by inducing a protective Ab response as well as eliciting memory B cells that are poised to respond rapidly to a secondary exposure. However, when the antigen used to recall a previously primed response differs from the original priming antigen as is the case with rapidly mutating viruses such as influenza virus or HIV, this complicates the recall response.

Shifting the antibody response

Early influenza studies suggest that recalling previously elicited influenza specific B cells that have only a low affinity for the new, mutated strain may be detrimental to the development of a higher affinity *de novo* response to the more recent strain. However, it has also been found that while *de novo* Ab responses specific for the secondary strain may be lower in individuals who have been pre-exposed to an antigenically different strain, the viral titers themselves are also lower in these pre-exposed individuals,

suggesting that additional protection and thereby a lower dose of antigen may be responsible for the reduction in *de novo* Ab titers (Kim et al., 2009).

Additional work has also shown that previous exposure can affect the Ab repertoire by shifting the focus of the Ab response to epitopes that are conserved between two sequentially infecting strains (Li et al., 2013; Wrammert et al., 2011). This is thought to be due to MBC clones responding more efficiently and therefore outcompeting naïve B cell clones that are specific for newly formed epitopes (Hebeis et al., 2004; Liu et al., 1995; Martin and Goodnow, 2002; Tangye et al., 2003). Interestingly, we found that the majority of the mAbs that we produced in PR8 pre-exposed mice were specific for the Sb and Sa sites on the distal head of the influenza HA in both the mice boosted with the PR8 strain or the antigenically distinct S12a strain. This suggests that shifting immunodominance hierarchies may require more extensive or precise alteration of the Ab footprint or additional exposures, as the antigenically drifted strain could recall previously elicited clones of relatively low affinity. Shifting Ab immunodominance to highly conserved sites is the target of several experimental vaccine regimens designed to broaden and increase protection against rapidly mutating and diverse viral populations (Ellebedy et al., 2014; Krammer et al., 2013; Wei et al., 2010). Thus we see that our goals have not changed much from the time of the Peloponnesian war when many hoped their past infection would protect them from a broader array of disease. Understanding how Ab specificity changes after a secondary response to an altered antigen will help us better understand not only how these changes may affect protection, but also inform future vaccine design.

Here we observe how Ab fine specificity changes early in the secondary response to an antigenically drifted virus with a heavily mutated HA molecule. We found

that exposure to a drifted strain increased the proportion of Abs that were cross-reactive between the two strains, but rather than focusing the Ab response on highly conserved epitopes in the stalk or head region of the HA, vaccination with the antigenically drifted S12a strain recalled Ab specificities that had slightly altered footprints in the variable regions of the HA head. This is in contrast to other published studies that elicited Abs to the highly conserved HA stalk domain (Krammer et al., 2013; Malherbe et al., 2011; Wang et al., 2010; Wei et al., 2010). However, our study differs from these in that we sequentially exposed mice with virus that had an intact but mutated HA head domain of the same HA subtype, and we examine the response after a second exposure. This suggests that the head domains may need to be more structurally distinct or that more repetitive vaccination may be required to elicit HA stalk-specific Ab responses. In our system, many of the Abs recalled at this early stage by S12a vaccination had a high affinity for the priming PR8 strain, but a low or immeasurable affinity for the S12a strain. This was in contrast to vaccination with a completely unrelated influenza strain such as B/Lee/1940, or an influenza strain that expressed an H3 HA (J1, A/HK/1/68 HA) which failed to recall PR8-specific cells into a secondary response despite eliciting new non cross-reactive Abs. This suggests that a certain degree of cross-reactivity is required, the threshold of which is still unknown.

Unexpected protection is elicited by low affinity antibodies

We found that B cell clones with very low to immeasurable affinity to the boosting strain can be recalled in secondary responses and that these clones may provide early protection *in vivo* to the boosting strain. This demonstrates the sensitivity of the BCR in recognizing antigen, and also the ability of soluble Abs to protect even with low affinity binding. How these Abs manage to neutralize virus despite their low affinity is yet to be

elucidated. Studies have shown that *in vivo* components of the immune system such as complement and Fc receptors enhance influenza-specific Ab mediated protection, particularly for Abs that are broadly cross-reactive or fail to neutralize *in vitro* (Feng et al., 2002). Several mouse models can be used to determine the role of these components in enhancing the low affinity Ab mediated protection evidenced in our work. While we transferred our mAbs into WT BALB/c mice, mice that have particular Fc receptor genes knocked out (such as FcRγ-/-, FcγRI-/-, FcγRIIb-/-, FcγRIII-/-, FcγRIV-/-, or FcRn-/- mice) can be used to test the contribution of particular receptors on the neutralization capability of specific Abs. In addition, recombinant mAbs can be engineered with Fc region mutations that abrogate or enhance binding of the Fc region to particular Fc receptors (DiLillo et al., 2014). Complement component C1q can also affect influenza virus-specific Ab mediated neutralization (Feng et al., 2002). The effect of C1q on Ab mediated neutralization can be tested by *in vitro* neutralization assays with purified C1q protein or using a C1q knockout mouse (Warren et al., 2002).

In addition to providing early protection, the recall of OAS Abs in a secondary response may have additional benefits. During a primary Ab response, autoreactive B cell clones are negatively selected while antigen-specific B cell clones are positively selected (Hande et al., 1998). While OAS clones may still reenter germinal centers and gain additional somatic mutations that increase their degree of autoreactivity, OAS clones which have been previously positively selected for their antigen-specificity may outcompete potentially autoreactive naïve clones. For instance, approximately half of the Cb-specific clones in the primary response to PR8 virus in BALB/c mice are encoded by a single Vk gene and one of two closely related VH genes (Kavaler et al., 1990). This group of B cell clones is excluded from the germinal center and does not form a memory

response (Kavaler et al., 1991; Rothaeusler and Baumgarth, 2010). Of note, one mechanism of inducing self tolerance is to exclude autoreactive B cell clones from the B cell follicle (Cyster et al., 1994; Mandik-Nayak et al., 1997). This particular group of B cell clones is not evident after secondary exposure, potentially due to it being outcompeted by recalled MBCs that are poised to respond rapidly to antigen (Kavaler et al., 1991). Recall of cross-reactive and OAS clones from the MBC repertoire may limit the activation of potentially autoreactive B cell clones in a similar manner as its exclusion of this particular set of Cb-specific B cell clones that are excluded from the memory response for an as yet unknown reason.

The effect of previous exposure on *de novo* antibody responses

We found that while S12a vaccination was able to boost a PR8-specific Ab response, this did not come at the cost of S12a-specific Ab titers. While our attention has been focused on the PR8-specific Abs recalled by S12a vaccination, we also isolated mAbs that were S12a-specific with immeasurable binding for PR8. These mAbs, isolated 3 days after S12a exposure, were all IgM isotype Abs. We hypothesize that these are early primary response Abs arising from previously naïve B cell clones. Due to the limited number of primary clones that we isolated at this early time point, we did not fully map the binding footprints of these mAbs. However, future studies should determine if the primary S12a-specific Ab repertoire is significantly affected by previous exposure to an antigenically distinct influenza virus. This could be done by expanding the panel of suspected primary S12a mAbs elicited in PR8 strain pre-exposed mice and comparing the binding footprints with S12a-specific primary mAbs elicited in previously naïve mice. Should there be a difference in specificity of Abs from PR8 strain pre-exposed mice, this could be due to competition for antigen or due to epitope shielding by

soluble Abs. Epitope shielding has been predicted to modify the fine specificity of the Ab response in a number of disease models (Tsouchnikas et al., 2015). To test whether competition for antigen affects the fine specificity of the response, one would first determine if there is an antigen dose dependent effect. As an additional control, one should compare S12a "primary" hybridomas from mice pre-exposed to the J1 strain which has a very distinct H3 HA and fails to recall PR8-specific clones as S12a can. Finally, one can analyze the "primary" S12a Ab repertoire in PR8 virus pre-exposed transgenic mice that fail to secrete soluble Ab to determine the effect of influenza virus-specific soluble Ab on the development of a *de novo* primary response to an antigenically drifted strain. These experiments would elucidate the potential effects of previous influenza virus exposure on the Ab repertoire developed in response to a novel, antigenically drifted influenza strain.

Maintaining diversity in the memory B cell repertoire

Our studies show that boosting sequentially vaccinating mice with antigenically drifted strains of influenza shaped a repertoire that included not only highly cross-reactive B cell clones, but also B cell clones that appeared to be specific for a single strain. This raises the question of how a diverse influenza-specific repertoire is maintained. We know that influenza-specific Abs can last a lifetime in humans, with Abs specific for the 1918 influenza pandemic strain still measurable in the very elderly (Krause et al., 2010). In fact, these Abs appear to be protective as evidenced by the protection of the elderly to the 2009 pH1N1 strain that exhibited some cross-reactivity to the 1918 pandemic strain (Ikonen, 2010). Despite this, new influenza-specific Abs are frequently acquired. There has been evidence that there is competition for a plasma cell niche in the bone marrow that provides survival signals to long-lived plasma cells that

periodically makes room for new plasma cells while still maintaining memory (Radbruch et al., 2006). Our studies show that the S12a strain can recall clones not only specific for itself but also those clones that have a low affinity for S12a and bind predominantly to a previously encountered strain. Similar evidence has been found in humans where recent influenza strains are shown to boost the titers of previously circulating strains (Fonville et al., 2014). This may be an important mechanism to broaden the influenza repertoire and enhance protection to future strains.

In addition, recent research suggests that the majority of MBCs exit the germinal center earlier than most long-lived plasma cells (Weisel et al., 2016). This suggests that although some memory cells are highly somatically mutated and may be recalled to diverse strains, many MBCs may not be as strain-specific as their highly mutated long-lived plasma cell relatives. This could impact the immune repertoire as highly specific long-lived plasma cells can provide protection in the form of secreted Ab, while the influenza-specific MBC population may remain less mutated but poised to rapidly mutate in the necessary direction to create strain-specific immunity.

New epitopes are potentially elicited by sequential exposure

While many mAbs have been mapped to the surface of HA by mutant selection, the antigenic sites previously described are by no means complete representations of potential epitopes. As most antigenic site determination has relied on analysis of viral mutants, residues that are functionally constrained and residues bound by non-neutralizing Abs that don't positively select for viral mutants are potentially overlooked. Therefore, the previously described antigenic sites likely represent the binding sites of the dominant neutralizing Abs, but not necessarily non-neutralizing Abs or Abs that bind

to highly conserved sites. These neglected groups can have important consequences for the immune response. Abs that bind to highly conserved sites are of particular interest as they have the potential to be more broadly cross-reactive and may therefore protect against a wider range of antigenically drifted strains of influenza.

Non-neutralizing Abs may also play an important role in susceptibility to infection. Little is known regarding the proportion of non-neutralizing Abs that is elicited in response to vaccination, infection, or sequential exposure. To determine the footprints of non-neutralizing antibodies, one could follow one of two approaches. First, one could introduce mutations throughout the HA, not only in those sites previously isolated after incubation with neutralizing antibodies, and test reactivity of non-neutralizing Abs to this expanded panel. This would however exclude those non-neutralizing Abs that bind to epitopes that are highly conserved due to functional constraints. Second, one could co-crystallize Abs with HA to determine the binding site of non-neutralizing Abs that bind to a highly conserved site. Such non-neutralizing Abs could be physiologically important as they could be recalled after sequential exposure without contributing to neutralization.

While the majority of both the PR8 strain-specific and cross-reactive mAbs that were elicited in our experiments bound to previously defined regions of the HA head, a number of mAbs bound to all of the viral mutants in our antigenic map panel. As our viral mutant panel is not exhaustive, these mAbs may bind to previously defined epitopes that are not addressed by our panel, or these mAbs may require more than one mutation in their footprint to abrogate binding. However, there is also the possibility that these mAbs bind outside of the previously dominant antigenic sites. Preliminary results using recombinant chimeric HA suggest that the unmapped mAbs in our studies are not HA stalk binding mAbs. A number of approaches can be used to define the presently

unclear binding footprint of these mAbs. PR8-specific mAbs that fail to bind S12a can be tested by ELISA against a panel of sequential mutants that contain between 1 and 13 mutations present in S12a to determine which of the mutations in the S12a strain abrogate binding. This will be particularly informative as it may be necessary for several mutations to be in place before the mAb fails to bind. Cross-reactive mAbs that bind to both the PR8 and S12a strains can be incubated with virus to isolate viral mutants that abrogate mAb binding to determine the possible locations of the binding footprint. This footprint could then be tested and expanded by additional ELISAs using viruses engineered to have mutations in those regions. Should these mAbs fail to neutralize virus *in vitro*, it will not be possible to isolate viral escape mutants in this manner. Instead, these mAbs can be tested against a panel of antigenically drifted viral isolates that have circulated since the PR8 strain was first isolated in 1934 to determine if any mutations have been randomly acquired throughout time that abrogate binding of these cross-reactive mAbs.

A potential mechanism for OAS

We have found that even PR8 strain-specific clones that have very low affinity for the S12a strain are recalled into a secondary Ab response following an S12a vaccination. Many of these clones have immeasurable affinity by ELISA. However, previous studies have shown that the sensitivity of the BCR can be much greater than that of soluble Ab (Lingwood et al., 2012). While the somatically mutated soluble Abs tested have measurable affinity for antigen, soluble Abs using the germline sequence do not. The germline Abs tested do however bind to antigen when expressed on the cell surface as BCR. We hypothesize that S12a virus immunization is able to recall PR8 strain-specific Abs despite their low affinity for the S12a strain because the BCR has an

increased sensitivity to the S12a virus HA in comparison to soluble Ab. Preliminary results in our laboratory have shown that plate-bound PR8-specific mAbs elicited in PR8 strain pre-exposed mice boosted with the S12a strain have a higher relative affinity for S12a virus HA than soluble mAb. This suggests that the conformation of Ab on a surface can increase the avidity of PR8 strain-specific mAbs for S12a virus. To test this hypothesis more meticulously in a more physiological setting, PR8 strain-specific mAbs with immeasurable S12a-specific affinity can be artificially expressed on the cell surface and binding to soluble antigen can be assessed by fluorescent antigen or by calcium indicator. This will indicate whether PR8 strain-specific mAbs recalled by S12a virus vaccination bind to S12a virus when expressed in a membrane bound form. This is of significance to the field of B cell immunity as it calls into question the sensitivity and comprehensiveness of analyses that measure the Ab repertoire using soluble Abs.

The effect of previous influenza virus exposure on influenza virus susceptibility

While our studies in a mouse model highlight how the fine specificities of Abs change after sequential exposure and outlines a potential protective role for OAS Abs, humans are continuously exposed to antigenically drifted strains of influenza that have varying degrees of similarity. In 2009, a new pH1N1 strain quickly replaced the previously circulating sH1N1 strain. The pH1N1 strain has a highly dissimilar head domain compared to previously circulating sH1N1 strains and has continued to accumulate mutations over time.

During the 2013-2014 influenza season, middle-aged adults were found to have an increased influenza disease burden. Studies have shown that different age groups have different influenza-specific Ab repertoires. We hypothesize that the age-specific

increase in susceptibility in the 2013-2014 influenza season is due to a recently acquired mutation that abrogates Abs prevalent in middle-aged adults due to their unique pre-exposure history. We find that human serum samples from middle-aged adults are more likely to exhibit reduced binding and neutralization against a mutant virus that contains the recently acquired K166Q HA mutation. mAbs that fail to bind to K166Q HA mutant influenza virus also fail to protect mice against infection with a K166Q HA mutant virus in an *in vivo* passive transfer experiment. These results suggest that an Ab repertoire focused on this newly mutated region negatively affects protection against infection.

We hypothesize that the K166 HA-specific Abs affected by the recent K166Q HA mutation are expressed by B cells that are the progeny of B cells previously elicited by sH1N1 strains. This is supported by analysis of human serum samples collected before and after immunization with the 2009 pH1N1 strain. This analysis shows that vaccination of middle-aged adults with the 2009 pH1N1 strain elicits an Ab response in some individuals that has reduced binding to a pH1N1 strain engineered to have a K166Q HA mutation. In addition, pH1N1 infected ferrets previously infected with a sH1N1 strain that circulated in the early 1980s (when middle-aged individuals were young) are more likely to have an Ab response that exhibits reduced binding to the new K166Q HA mutant strain. Taken together, these results suggest that while previous exposure shifts the Ab response to epitopes conserved between sequentially circulating viral strains, the shift in epitope immunodominance can affect susceptibility should a previously conserved region acquire a mutation by continued antigenic drift.

Updating the antibody repertoire

Our work suggests that a new mutation has been acquired by circulating pH1N1 strains that abrogates binding of Abs commonly prevalent in middle-aged adults. We therefore recommend that the H1N1 component of the influenza vaccine be updated to include a currently circulating strain that has a K166Q HA mutation. However, we do not know whether individuals vaccinated or infected with a Q166 HA viral strain will produce protective Abs to this epitope, or whether the antibody response will be shifted to a different region of the HA. To test whether an updated strain would elicit Q166-specific Abs in these individuals, serum samples collected before and after vaccination or natural infection with a Q166 HA viral strain should be analyzed by HAI assay and by *in vitro* neutralization assay. This will illustrate how efficiently exposure to a highly similar influenza strain with a relevant antigenic mutation can update an Ab repertoire that is focused on a recently mutated site.

Understanding B cell immunodominance

We propose that the H1N1 component of the influenza vaccine be updated despite the fact that more conventional surveillance techniques that use primary ferret sera fail to determine that the K166Q HA mutation is antigenically relevant. We suggest that this is due to the fact that the primary Ab response in previously naïve ferrets infected with the pH1N1 strain is focused on a region of the HA encompassing HA residues 156, 157, and 158 that is not affected by a mutation at residue 166 (Li et al., 2013). Why epitopes containing residues 156-158 of the HA are immunodominant for the ferret Ab response to the pH1N1 strain is currently unknown. In fact, the mechanism of B cell immunodominance is largely unexplained. Unraveling this mystery would be of

great value to future vaccine design and the understanding of the B cell response to infection, autoimmunity, and tumor antigens.

Immunodominance is not permanent and can be shifted from one epitope to another. In fact, shifting humoral immunodominance to subdominant but broadly neutralizing epitopes is the strategy of a number of experimental universal influenza vaccines. However, although it has been possible to shift the immune response to conserved epitopes like the HA stalk, this has been a transient effect as re-infection or immunization of an individual tends to shift the Ab response back to the dominant HA head domain epitopes (Andrews et al., 2015; Ellebedy et al., 2014). It has been suggested that subdominant epitopes only become dominant when they are recalled in the absence of competition from previously elicited clones to typically dominant epitopes. This would explain why previously subdominant HA stalk-specific B cell clones become more prevalent in humans who are infected or immunized with the 2009 pH1N1 strain which has an extensively mutated HA head domain in comparison to previously circulating sH1N1 strains (Wrammert et al., 2011). This is also hypothesized to be the reason why sequentially immunizing mice with HA that have conserved HA stalk domains but highly diverse head domains shifts the Ab response towards the stalk domain (Ellebedy et al., 2014; Krammer et al., 2013). However, while previously elicited B cell clones specific for subdominant epitopes may be temporarily dominant, the repertoire remains diverse. Once exposed to an HA head domain, new B cell clones specific for those dominant epitopes arise and these can once again outcompete B cell clones that are specific for subdominant epitopes. This would explain why the HA stalk-specific response in individuals who are infected or immunized with the 2009 pH1N1

strain lose immunodominance against the HA stalk domain after a second exposure to the pH1N1 strain (Andrews et al., 2015).

If one could limit the repertoire to broadly cross-reactive clones, those clones that are specific for the dominant but more strain-specific epitopes would not outcompete the subdominant broadly cross-reactive clones. However, to make this a possibility, one would not only need to create a vaccine that lacks the previously dominant epitopes, but it would also have to be so strongly neutralizing that any natural exposure to circulating influenza strains would fail to mount a *de novo* Ab response to the dominant but variable head domain as any Ab response to more dominant epitopes would eventually outcompete the more broadly neutralizing subdominant clones. This has been attempted in the form of "headless" HA vaccines (Steel et al., 2010; Uranowska, 2014; Yassine et al., 2015). The HA protein is initially expressed as an HA0 protein which is later cleaved into the HA1 and HA2 subunits. These two subunits are bound together by disulfide bounds. The HA head domain is encoded entirely by the HA1 region, while the HA stalk domain is encoded by both the HA1 and HA2 subunits. Should these subunits separate, the head domain remains intact while the stalk domain is incomplete. HA1 subunits have been shown to induce an Ab response, whereas the HA2 subunit has not (Khurana et al., 2011b; Sguazza et al., 2013). This could potentially affect immunodominance of the head domain and reduced immunogenicity of the stalk domain should the disulfide bonds be cleaved *in vivo* (Hogg, 2003). The headless HA constructs that have been artificially created contain the HA2 subunit and the region of HA1 that contributes to the HA stalk domain while excluding the region that encodes the HA head domain. Interestingly, sera collected from mice immunized with "headless" HA failed to neutralize virus in *in vitro* neutralization assays, but these mice were protected against *in*

vivo challenge with a homologous virus (Steel et al., 2010). This contrast might be explained in light of recent results that demonstrate that many stalk-specific Abs require Fc receptor binding for neutralization activity (DiLillo et al., 2014). However, while these headless HA constructs initially elicited an Ab response that was more broadly cross-reactive in a subset of vaccinated mice, whether this remained true after viral challenge remains unaddressed. Based on our current experience with pH1N1 infections, this appears to be unlikely, underscoring the need to more fully understand the mechanisms of B cell immunodominance.

Concluding Thoughts

The observation of OAS has been paradoxical to the theory of clonal selection as an antigenically drifted strain of influenza virus recalls B cell clones specific for a previously circulating strain that appear to fail to bind to the new, drifted strain. However, we know that a certain degree of cross-reactivity is required. While we might expect antigen to induce the recall of the strongest affinity B cell clones, antigenically drifted strains of influenza can recall B cell clones that have extremely low affinity for the new drifted strain relative to the affinity to an older strain. While this can occur at the expense of mounting a *de novo* Ab response to the drifted strain in the case of sequential infection, in our experiments there is no detrimental effect on overall titers to the drifted strain after sequential vaccination with inactivated virus. In addition, the OAS Abs elicited after recall by a drifted strain can have a positive effect on protection and this can be further affected by somatic hypermutation. However, while we show that OAS Abs can contribute to a protective immune response, shifting the Ab response towards regions conserved between the previously experienced strains can potentially affect individual susceptibility to infection to further drifted strains. We show that an HA

mutation, K166Q, negatively affects binding of human sera of middle-aged adults and that a K166 HA-specific mAb isolated from a middle-aged adult fails to neutralize K166Q HA mutant viruses. This therefore highlights the pros and cons of shifting the Ab repertoire in response to sequential exposure to antigenically drifted strains of influenza virus (Figure 21). The challenge of future endeavors in this field will be to determine how to manipulate these repertoire changes to elicit the Abs of choice against not only influenza virus, but also other difficult targets, such as HIV and tumor antigens. Our ability to design more effective vaccines and antibody therapies will continue to improve as we put together piece by piece the puzzle that is Ab immunodominance and the ever-shifting Ab repertoire.

Figure 21

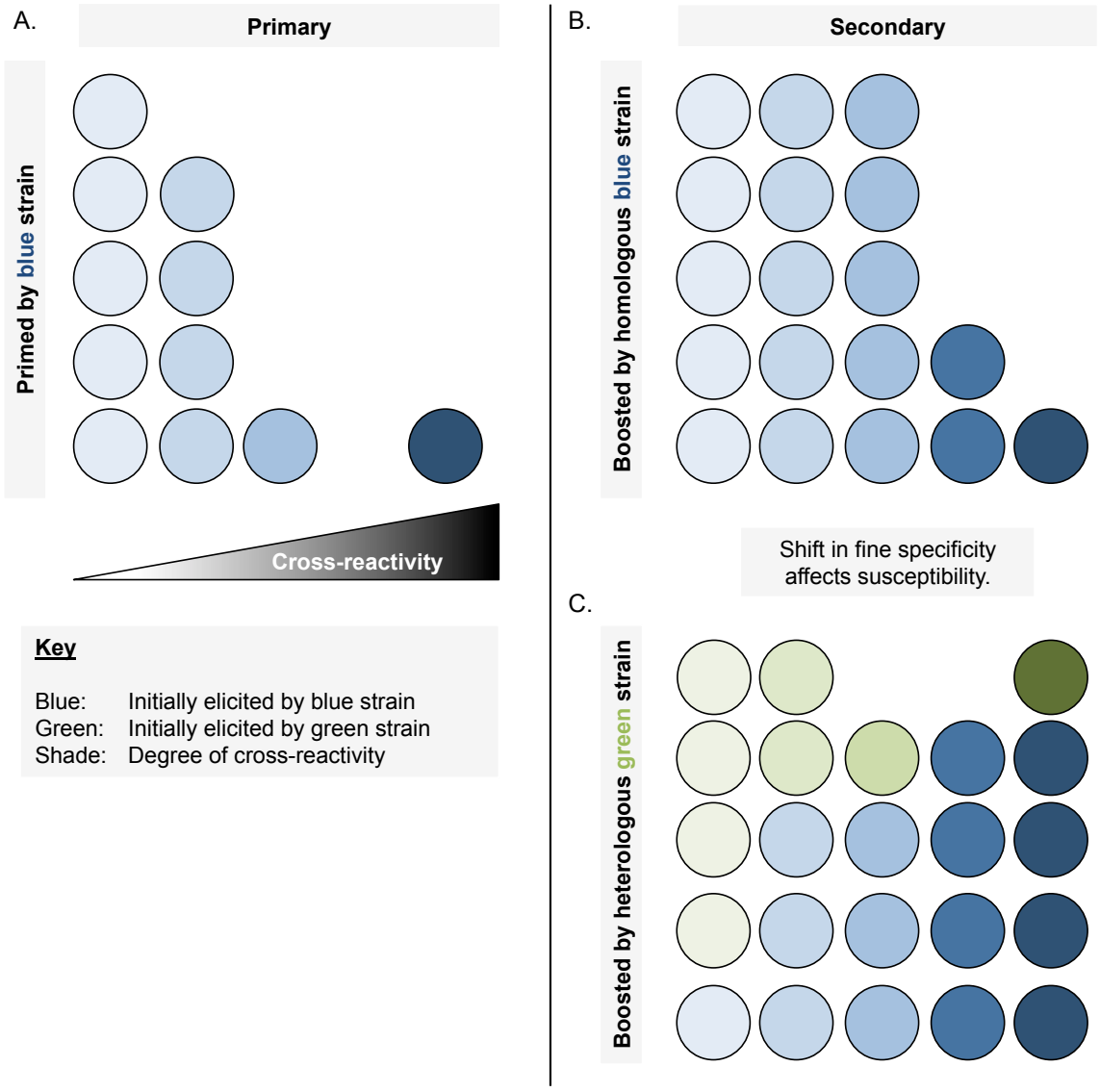


Figure 21. A schematic diagram of the effect of sequential exposure to antigenically drifted strains of influenza on the specificity and function of the Ab repertoire

(A) A primary HA-specific B cell response to a particular 'blue' strain elicits Abs that are predominantly strain-specific (represented by a light shade) and not highly cross-reactive

(represented by a darker shade). (B) After a secondary response to the same 'blue' strain, Ab specificity may shift to other epitopes, but the B cell clones remain predominantly strain-specific, with only a slight increase in the degree of cross-reactivity to antigenically drifted strains. (C) In contrast, the fine specificity of B cell clones elicited by heterologous exposure differs from those elicited by a homologous recall response. Individuals who are pre-exposed to the 'blue' strain and then boosted with an antigenically distinct 'green' strain elicit not only a *de novo* Ab response against the 'green' strain, but also recall Abs previously elicited by the 'blue' strain that have varying degrees of cross-reactivity. There is an increased proportion of B cell clones expressing highly cross-reactive Abs, but also recall of B cell clones expressing Abs with a higher affinity for the 'blue' strain than the 'green' strain. Many of these Abs with a higher affinity to the priming 'blue' strain are protective against the 'green' strain. However, the shift in Ab fine specificity after sequential exposure not only affects cross-reactivity and protection against the 'green' strain, but may affect susceptibility to a novel antigenically drifted strain that has a mutation that abrogates binding of these Abs.

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